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Attorney Docket No. 990360/LH

Asst. Commissioner for Patents
Washington, D.C. 20231

Transmitted herewith for filing UNDER SMALL ENTITY STATUS is the patent application of

Inventor(s): Masataka KINJO

Title: "METHOD AND APPARATUS OF ANALYZING TARGET NUCLEIC ACID"

Priority Claim (35 U.S.C. 119) is made, based upon:

Japan No. 10-347493 December 7, 1998
Japan No. 10-353254 December 11, 1998

Enclosed herewith are:

- ☒ Specification (Description, Claims, Abstract): Pages 1 - 74 ; Number of claims 1 - 37
- ☒ Declaration and Power of Attorney ☒ executed; ☐ unexecuted (supplied for information purposes)
- ☒ 8 Sheets of drawings, Figures 1 - 16 ☒ Formal ☐ Informal
(FIGS. 4A, 4B, 11A AND 11B ARE BLACK & WHITE PHOTOGRAPHIC DRAWINGS)
- ☐ Assignment and "Patents" Recordation Form Cover Sheet (PTO-1595) & \$40. RECORDATION FEE.
- ☐ Certified copy (ies) of priority document(s) identified above
- ☐ Information Disclosure Statement; ☐ Form PTO-1449
- ☒ Preliminary Amendment (TO CORRECT AN ILLEGAL MULTIPLE DEPENDENCY)
- ☒ Verified Statement(s) Claiming Small Entity Status
- ☒ Receipt Postcard

	Number Filed		Number Extra	Rate	Calculations
Total Claims	73	-20 =	53	x \$ 9.00	= \$ 477.00
Independent Claims	3	- 3 =	0	x \$39.00	= \$
MULTIPLE DEPENDENT CLAIMS				+\$130.00	= \$ 130.00
				BASIC FEE	\$ 380.00
				Total of above Calculations	\$ 987.00

To the extent not tendered by check, authorization is given to charge any fees under 37 CFR 1.16 and 1.17 during pendency of the application, or to credit any overpayment, to Deposit Account No. 06-1378. Duplicate copy of this letter is enclosed.

FRISHAUF, HOLTZ, GOODMAN, LANGER & CHICK, P.C.

By: Leonard Holtz, Reg. No. 22,974

11/10/98

LH:bv

Figure 1 displays 12 horizontal gel electrophoresis images, each showing a series of DNA bands. The samples are labeled on the left: 1. 100bp DNA ladder, 2. 100bp DNA ladder, 3. 100bp DNA ladder, 4. 100bp DNA ladder, 5. 100bp DNA ladder, 6. 100bp DNA ladder, 7. 100bp DNA ladder, 8. 100bp DNA ladder, 9. 100bp DNA ladder, 10. 100bp DNA ladder, 11. 100bp DNA ladder, 12. 100bp DNA ladder. The bands are numbered 1 through 12 at the top of each gel.

**IN THE UNITED STATES PATENT
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Patents, Washington, D.C. 2
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Applicant or Patentee: Masataka Kinjo
Serial or Patent No.: _____ Atty. Dkt. No.: _____
Filed or Issued: _____
For: METHOD AND APPARATUS OF ANALYZING TARGET NUCLEIC ACID

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS (37 CFR 1.9(f) and 1.27(b)) - INDEPENDENT INVENTOR**

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled METHOD AND APPARATUS OF ANALYZING TARGET NUCLEIC ACID described in

- (X) the specification filed herewith
() application serial no. _____, filed _____
() patent no. _____, issued _____

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below.

- (X) no such person, concern, or organization
() persons, concerns or organizations listed below*

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27)

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF INVENTOR <u>Masataka Kinjo</u>	NAME OF INVENTOR	NAME OF INVENTOR
Signature of Inventor <i>Masataka Kinjo</i>	Signature of Inventor	Signature of Inventor
Date <u>May. 20. 1997</u>	Date	Date

TITLE OF THE INVENTION

METHOD AND APPARATUS OF ANALYZING TARGET NUCLEIC ACID

BACKGROUND OF THE INVENTION

5 The present invention relates to a technique for
analyzing a nucleic acid molecule present in a test
sample, and more particularly, to a method for
specifically measuring nucleic acid molecules amplified
by applying a nucleic amplification method. The
present invention is applied to a detection method in
10 which measurement data with respect to a single nucleic
acid molecule is valuable. Accordingly, the present
invention can be effectively applied to any cases for
the purpose of obtaining quantitative information
regarding the number of target nucleic acid molecules.

15 Determination and detection of nucleotide
sequences and measurement of molecular weights
regarding nucleic acids such as DNA and RNA, are the
most important analytical means in biochemical and
molecular biological researches, and recently, have
20 also become an important tool in gene diagnosis and
gene therapy. Generally, in the case where these
analytical means are applied to biological test samples
such as blood, urine, cerebrospinal fluid, and saliva,
the analysis is performed after amplifying nucleic acid
25 molecules present in an extremely small amount in the
test sample to an appropriate concentration, in order
to increase accuracy in the analysis. For example,

in PCR (polymerase chain reaction), which is a very important gene amplification method, two types of primers work together in a plurality of amplification cycles. An amplified gene through appropriate

5 amplification cycles is finally detected by subjecting the amplified sample to gel electrophoresis to separate them into fractions based on chain length (molecular weight), and then, determining the presence and absence of a fraction corresponding to a specific chain length.

10 In the PCR, a target nucleic acid is treated in the presence of DNA polymerase serving as a nucleic acid synthase, primers for initiating replication, and four types of bases (dATP, dGTP, dTTP, dCTP) serving as substrates for use in replication, under conditions

15 in which a polymerizing reaction of the substrate molecules can take place. It follows that a complementary chain is synthesized by using a single-stranded DNA derived from the target nucleic acid as a template to form a double stranded DNA (dsDNA), resulting in

20 amplification of the nucleic acid. As described, the PCR product amplified by co-working of two types of primers always forms a double stranded DNA (dsDNA).

 The hybridization reaction using an oligonucleotide probe having a specific nucleotide

25 sequence is used for determining a nucleotide sequence of the PCR product, namely, dsDNA. However, the hybridization reaction using the oligonucleotide probe

has such problems that non-specific hybridization easily takes place because quick annealing must be repeated, and further, both DNA chains resemble the probe in nucleotide sequence.

5 As a quantitative method for measuring a total amount of dsDNA after amplified by the PCR method, some methods have been proposed hitherto. For instance, one of the methods is as follows. PCR is effected in a predetermined number of cycles using a sufficient
10 amount of a PCR primer labeled with a marker molecule such as a fluorescent dye. The PCR product obtained through the predetermined cycles is subjected to electrophoresis to separate into a primer elongated by polymerase reaction and a free primer not elongated
15 (Bound/Free separation). After the bound/free separation, a total number of fluorescent molecules is counted by a fluorometer. Also known is another method using, as the fluorescent marker, an intercalator (eg. acridine orange, thiazole orange, oxazole yellow, etc.)
20 which cannot emit fluorescence until it intercalates into the dsDNA during the amplification process. In this method, it is possible to measure the intensity of fluorescent which is emitted from dsDNA hybridized in a test solution only, without effecting the Bound/Free
25 separation.

 In the methods described above which measure a total fluorescent amount obtained from a whole test

solution, the primers are used in a sufficient amount so as to cause no shortage of the primer during the nucleic acid amplification process of predetermined cycles regardless of an initial concentration of a target nucleic acid molecule. However, in the amplification reaction in the PCR method, various reaction curves are obtained depending upon not only the number (or amount) but also types of target nucleic acid molecules present in a test sample. It is therefore difficult for a conventional analytic method, which measures a total amount of the marker molecules attached to the amplified nucleic acids, to quantitatively monitor the reaction process. In particular, in the conventional PCR method, a signal derived from the free labeled molecules is eliminated and only a signal from the labeled primer hybridized with the target nucleic acid is measured, by removing free labeled molecules through the Bound/Free separation or by employing the intercalator. It is therefore impossible to monitor whether a particular free primer molecule is hybridized or not. Accordingly, in the conventional target nucleic acid analysis, it is impossible to perform quantitative analysis in the presence of a free primer molecule. Furthermore, since a total amount of fluorescence of the whole test solution is measured, the amount of the test solution must be increased in order to improve the sensitivity of the measurement.

In addition, since the measurement cannot be performed until a nucleic acid is sufficiently amplified, it is almost impossible to detect a target nucleic acid in an earlier stage of amplification. More specifically, it is difficult to perform quantitative analysis for determining the number (or amount) of the target nucleic acids in the mid-stage of the PCR amplification process, let alone in the earlier stage of the process.

On the other hand, a technique has been lately developed for accurately determining micro-motions of individual target molecules by measuring fluctuation motion of the target molecule in fluid and applying an autocorrelation function. The method of this type is called as a fluorescence correlation spectroscopy (referred to as "FCS" hereinafter) since a fluorescent marker molecule is measured by an optical instrument. An arithmetical operation using the FCS for processing data regarding a biological material are disclosed by Kinjo et al., in which FCS is employed in a hybridization reaction between a labeled nucleic acid probe and a target nucleic acid molecule (Kinjo, M., Rigler, R., Nucleic Acids Research, 23, 1795-1799, 1995).

As a method for detecting a target gene by use of FCS, some reports have been recently provided. According to the report made by Oehlenschlaeger, F. et al. (Proc. Natl. Acad. Sci. USA, 93, 12811-12816, 1996), it is demonstrated that NASBA (Nucleic Acid Sequence

Based Amplification) combined with FCS is an effective detection method for diagnosing HIV (Human Immunodeficiency Virus) in a serum. The NASBA method, which is a modified PCR method, requires an additional primer labeled with fluorescence as a probe other than two primers described above in the presence of three enzymes, i.e., T7 RNA polymerase, reverse transcriptase, and RNaseH.

As a more simplified method than the NASBA method, an amplified probe extension (APEX) using FCS has been reported from the same group (Walter, N. G. et al., Proc. Natl. Acad. Sci. USA, 93, 12805-12810, 1996). Even in the simplified method, an additional primer labeled with fluorescence is also required as a probe, besides a set of a forward primer and a reverse primer. In an experiment of APEX, change in autocorrelation function value with time is observed after 26 cycles.

However, in both the NASBA method and the APEX method, since an additional labeled probe is required besides the materials for use in general nucleic acid amplification, reaction conditions becomes complicated, making it difficult to stably control an accuracy of analysis. Furthermore, since the probe molecule is labeled with a single fluorescent marker, a signal derived from a labeled free probe significantly reduces an S/N ratio. As a result, the detection sensitivity tends to decrease as the initial concentration of the

target nucleic acid molecule decreases. Furthermore, many cycles of PCR reactions must be performed to ensure the detection.

BRIEF SUMMARY OF THE INVENTION

5 The present invention was made in view of the aforementioned circumstances surrounding nucleic acid detection methods.

10 An object of the present invention is to provide a method of accurately and quantitatively detecting an amount of a target nucleic acid molecule by considering a labeled free molecule also as a measurement target. Another object of the present invention is to provide a method of quantitatively monitoring and measuring a PCR amplification reaction. Still another object
15 of the present invention is to provide a method of quantitatively detecting a nucleic acid molecule even if the copy number is low. A further object of the present invention is to provide a method of detecting the presence and absence of the target nucleic acid
20 molecule even if a small amount of test sample is available. A still another object of the present invention is to provide a method for detecting the presence and absence of the target nucleic acid in an earlier stage of the amplification process. A still
25 further object of the present invention is to provide a method of measuring a nucleic acid molecule, capable of investigating a PCR amplification process

at a molecular level.

To attain the aforementioned objects, according to a first aspect of the present invention, there is provided a method of analyzing a target nucleic acid by applying a nucleic acid amplification reaction to a test solution, the method comprises the steps of:

performing a nucleic acid amplification reaction of the target nucleic acid using a test solution containing a primer, substrate molecule at least one of which is labeled with a marker molecule capable of generating a detectable signal, a nucleic acid synthase, and a target nucleic acid molecule;

measuring a signal from the marker molecule in the test solution after initiation of the nucleic acid amplification reaction;

evaluating mobility of the labeled molecule in the test solution on the basis of the signal detected; and quantifying the target nucleic acid molecule on the basis of evaluation results.

According to a second aspect of the present invention, there is provided an apparatus of analyzing a target nucleic acid by applying a nucleic acid amplification reaction to a test solution, the apparatus comprises:

holding means for holding a test solution containing a primer, substrate molecules at least one of which is labeled with a marker molecule capable of

generating a detectable signal, a nucleic acid synthase,
and a target nucleic acid;

measuring means for measuring a signal from the
marker molecule after initiation of a nucleic acid
5 amplification reaction in the test solution;

evaluation means for evaluating mobility of the
marker molecule in the test solution on the basis of
the signal detected; and

data output means for outputting a evaluation
10 result obtained by the evaluation means as a quantifi-
cation data of the target nucleic acid molecule.

According to a third aspect of the present
invention, there is provided a method of quantitatively
analyzing a target nucleic acid molecule present in
15 a biological sample, comprising:

an amplifying step of amplifying the target
nucleic acid by using first and second primer molecules
having sequences which are complementary with two
discrete nucleotide sequence regions of the target
20 nucleic acid molecule respectively, at least one of
the first and second primers being labeled with a
detectable marker molecule and at least the number of
labeled primer molecules being known;

a measurement step of obtaining measurement data
25 regarding the labeled molecule by using at least a part
of a test solution which has been subjected at least
a single amplification step; and

a determination step of determining a number and a size of the target nucleic acid molecules on the basis of the measurement data.

Additional objects and advantages of the invention
5 will be set forth in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The objects and advantages of the invention may be realized and obtained by means of the instrumentalities and combina-
10 tions particularly pointed out hereinafter.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

The accompanying drawings, which are incorporated in and constitute a part of the specification, illustrate presently preferred embodiments of the invention,
15 and together with the general description given above and the detailed description of the preferred embodiments given below, serve to explain the principles of the invention.

FIG. 1 is a schematic view showing an example of
20 an apparatus for carrying out a method of the present invention;

FIG. 2A is a partially enlarged view of the apparatus shown in FIG. 1;

FIG. 2B is an enlarged schematic view showing
25 a micro-field of view in FIG. 2B;

FIG. 3 is a schematic diagram of asymmetric PCR according to the present invention;

FIGS. 4A and 4B are electrophoretic (agarose) photographs each showing characteristics of an amplified product (4 kb);

FIG. 5 is a graph showing the relationship between the marker concentration and the amount of products in PCR, obtained as a function of a percent ratio of Flu-dUTP;

FIG. 6 is a graph showing change of the autocorrelation function with time in a PCR reaction process;

FIG. 7 is a graph obtained by normalizing each of autocorrelation functions at zero time-lag;

FIG. 8 is a graph showing the number of amplified DNA molecules in a PCR reaction solution (5 μ L) versus the PCR cycle number;

FIG. 9 is a graph showing the number of DNA molecules after 20 cycles versus the initial template number;

FIG. 10 is a graph showing a typical fluorescence autocorrelation function;

FIGS. 11A and 11B are photographs each showing the results of agarose gel electrophoresis of an amplified target product (500 bp), performed after quantitative measurement by FCS:

FIG. 12 is a graph showing fluorescence autocorrelation functions in case that an initial template concentration before PCR in a reaction solution (25 μ L) is changed from 1.9×10^7 to 1.9 for investigating the

sensitivity of the method of the present invention;

FIG. 13 shows an average number of fluorescent molecules in an optical measurement field (a micro detection field) versus the initial template concentration shown in FIG. 12;

FIG. 14 is a graph showing the yield (%) of 500 bp product versus the initial template concentration;

FIG. 15 is a graph showing the initial template number present in a mixed solution (25 μ L) versus the yield (%) of each of PCR products obtained through different numbers of PCR cycle; and

FIG. 16 is a graph showing PCR productions per initial template DNA molecule obtained through different PCR cycles, the graph being derived by dividing the measurement data of FIG. 15 per numbers of initial template DNA molecules.

DETAILED DESCRIPTION OF THE INVENTION

Hereinafter, the present invention will be described in more detail.

In a preferred embodiment of the first and second aspects of the present invention, at least one of monomer nucleotides serving as substrates in the PCR reaction is labeled with a fluorescent marker, and this fluorescent-labeled monomer nucleotide is monitored.

To be more specific, the present invention has been made based on such a finding successfully demonstrated by image analysis rather than gel

electrophoresis that when a target nucleic acid is amplified by using, as one of monomer substrates, fluorescein-11-dUTP which is a monomer nucleotide labeled with a fluorescent dye molecule, the molecular weight of the target nucleic acid changes during the amplification process including the incorporation of the fluorescent monomer nucleotide.

Furthermore, the present invention has been made based on the finding that when the motion of the fluorescent dye molecule was evaluated by applying an autocorrelation function, quantitative results were obtained. Furthermore, the present invention is based on the finding that sensitivity was further improved without any adverse influence on measurement, by separating the labeled monomer nucleotide not incorporated into the amplification products during the nucleic acid amplification process, by using, for example, a gel filtration column.

According to a first aspect of the present invention, there is provided a method of analyzing a target nucleic acid by applying a nucleic acid amplification reaction to a test solution, the method comprises the steps of:

performing a nucleic acid amplification reaction of the target nucleic acid using a test solution containing a primer, substrate molecule at least one of which is labeled with a marker molecule capable of

generating a detectable signal, a nucleic acid synthase, and a target nucleic acid molecule;

measuring a signal from the marker molecule in the test solution after initiation of the nucleic acid

5 amplification reaction;

evaluating mobility of the labeled molecule in the test solution on the basis of the signal detected; and

quantifying the target nucleic acid molecule on the basis of evaluation results.

10 The step of performing the nucleic acid amplification reaction is well known to the skilled in the art, and the nucleic acid synthase may be a polymerase or a reverse transcriptase.

15 It is preferred that the measurement step include a step of measuring an amount (number) of the marker molecule present in a predetermined measurement region. It is further preferred that, in the measurement step, a moving amount of the marker molecules within a predetermined time interval be measured for a plurality
20 of time points. Furthermore, a step of removing the labeled substrate molecule not reacted in the nucleic acid amplification reaction may be included between the amplification reaction step and the measurement step.

25 It is preferable that the measurement is performed in such condition that the measurement system is not contact with the test solution, because the non-contact measurement not only improves sensitivity but also

enhances the reproducibility of the measurement results without reducing accuracy.

It is preferred that the evaluation step described above includes a step of converting a plurality of measurement data into statistical data which express a change in the moving amount of the marker molecule. Furthermore, the conversion step preferably includes a step of performing arithmetic operation applying an autocorrelation function.

Furthermore, it is preferred that the quantification step described above includes a step of determining the presence and absence of the marker molecule incorporated into the amplified products during the nucleic acid amplification process based on the evaluation results. Furthermore, it is preferred that the quantification step includes a step of determining the amount (or number) of the labeled nucleic acids incorporated into the amplified products during the nucleic acid amplification process, based on the evaluation results.

According to a second aspect of the present invention, there is provided an apparatus of analyzing a target nucleic acid by applying a nucleic acid amplification reaction to a test solution, the apparatus comprises:

holding means for holding a test solution containing a primer, substrate molecules at least one

of which is labeled with a marker molecule capable of generating a detectable signal, a nucleic acid synthase, and a target nucleic acid;

measuring means for measuring a signal from the marker molecule after initiation of a nucleic acid amplification reaction in the test solution;

evaluation means for evaluating mobility of the marker molecule in the test solution on the basis of the signal detected; and

data output means for outputting a evaluation result obtained by the evaluation means as a quantification data of the target nucleic acid molecule.

It is preferred that the measurement means described above have an optical system for performing measurement within a micro detection field restricted in a diffraction-limited region near a focal point.

Alternatively, the measurement means preferably have a microscope for attaining measurement in the micro detection field formed by a confocal optical system.

It is further preferable that the diffraction-limited region be formed by means of an aperture having an average diameter of $30 \pm 20 \mu\text{m}$. Furthermore, it is preferable that the diffraction-limited region be formed by means of an aperture having an average diameter of $20 \pm 10 \mu\text{m}$.

It is preferred that the micro detection field be nearly a cylindrical region having an average radius

200 \pm 50 nm and an average length on an optical axis of
2000 \pm 500 nm.

It is preferred that the evaluation means
described above comprises a means for storing a
5 plurality of measurement data obtained in a predeter-
mined time interval and an arithmetic operating means
for processing the plurality of measurement data stored
by applying an autocorrelation function. Furthermore,
it is preferred that the evaluation means comprise
10 a means for storing measurement data with respect to a
plurality of labeled molecules or the marker molecules
obtained in a predetermined measurement region, and
an arithmetic operating means for processing the stored
measurement data with respect to each of the labeled
15 molecules or the marker molecules by applying an
autocorrelation function.

It is further preferable that the data output
means described above comprise a conversion means
for converting the arithmetically processed results
20 obtained by applying the autocorrelation function
into statistical data which express a positional change
with time of each labeled molecule corresponding to
a plurality of monitoring data.

In the method according to the first aspect of
25 the present invention, at least one type of substrate
molecules which will be incorporated into the amplified
products during the nucleic acid amplification process

of the PCR is labeled with a marker molecule capable of generating a detectable signal. The substrate molecule, i.e., monomer nucleotide having such a marker molecule is maintained free during a hybridization between the target nucleic acid and the primer for PCR initiation. In the nucleic acid synthesis process following the hybridization, when the nucleic acid synthase works on the primer hybridized to the target nucleic acid molecule, the substrate molecules are sequentially incorporated into the primer for extending the primer to the amplified product. The marker molecule which is incorporated together with the substrate molecule into the amplified product during the nucleic acid amplification process, decreases in mobility in the test solution, as compared to the marker molecule bonded to the free substrate molecule.

Then, at least a part of the test fluid which contains amplified product from efficient PCR cycles is quantitatively taken as an aliquot in the holding means and subjected to the measurement of the marker molecule. The holding means for the test solution used herein may be a concave-form container such as a test tube, a well, or a Petri dish, or a flat-plate holder such as a slide glass. Depending on the situation, the test fluid may be held by directly adhering it as a drop on the surface of the measurement means. Alternatively, the test fluid contained in a PCR reaction container may be

subjected to the measurement as it is.

Any measurement means may be employed as long as it can measure a change in size of the molecule carrying the marker during nucleic acid amplification process. For example, gel electrophoresis, capillary electrophoresis, flow-cytometer for fluorescence measurement, image cytometer, and fluorescence microscopy or the like may be effectively used.

When the measurement step is performed in a three dimensional micro detection field, free micro-motion of the labeled molecule in the test fluid can be accurately determined while keeping its natural state corresponding to the results of PCR reaction. The micro detection field can be optically designed, for example, by converging light from a halogen lamp or by emitting light through an aperture having an extremely small average radius. In this case, it is preferable to use converged laser light.

When the micro detection field is formed by a confocal optical system in the measurement step, measurement data can be obtained with a deep depth of field. As a result, individual arbitrary marker molecules are always brought into focus in the detection field so that accurate positional data and fluorescent output data can be supplied to the measurement means.

If the micro detection field falls within the

diffraction limited region near a focal point, the individual marker molecules can be measured with a high S/N ratio.

5 If the diffraction limited region is formed by a pin hole having an average diameter of $30 \pm 5 \mu\text{m}$, measurement data of a small number of selected marker molecules can be efficiently obtained.

10 When the micro detection field is formed in nearly a cylindrical form having an average radius of $200 \pm 50 \text{ nm}$ and an average length on the optical axis of $2000 \pm 500 \text{ nm}$, it is possible to capture the free micro-motion of the marker molecule sighted in the measurement detection field.

15 In the present invention, speed of the marker molecule moving in and out of the micro detection field is measured by using, as an indication, increase/decrease or appearance/disappearance of output signal intensity from at least one marker molecule present in a predetermined space. Accordingly, the preferred
20 marker molecule to be tagged to the substrate molecule is that capable of outputting a constant signal at a plurality of measurement points. The preferred marker molecule includes luminescent material, fluorescent material, magnetic material, radioactive material and
25 the like. In particular, as the luminescent material and fluorescent material, a dye that emits luminescence or fluorescence for a long time may be preferable.

If the marker molecule having a luminous dye or a fluorescent dye is used, measurement of molecular level can be effected by using a simple optical system.

As the fluorescent dye, there are well known dyes such
5 as DAPI, FITC, rhodamine, Cy 3, Cy 3.5, Cy 5, Cy 5.5, Cy 7 and the like.

When mobility of the fluorescence molecule is measured in the measurement step, fluorescent light is received by a fluorescence measurement means such as
10 a photo-multiplier or a photo-diode. The fluorescence measurement means having a measurement mode capable of measuring a single photon is advantageous because it enables to measure individual fluorescent molecules.

It is possible to attain the accurate measurement
15 of each marker molecule in a more effective embodiment in which the fluctuation motion of the marker molecule in a fluid is measured in the measurement step. In the case where the fluctuation motion is measured, arithmetic operation may be preferably performed by
20 applying autocorrelation function. In particular, when fluorescent molecule is used as the marker molecule, it is preferred that fluorescence correlation spectroscopy (simply referred to as "FCS" hereinafter) be used.

In arithmetically operating the measurement data of
25 biological material by using FCS, reference can be made to a report by Kinjo et al. in which FCS is used in a hybridization reaction between a labeled nucleic acid

probe and a target nucleic acid molecule (Kinjo, M.,
Rigler, R., Nucleic Acids Research 23, 1795-1799, 1995).

An apparatus for performing FCS is schematically
shown in FIG. 1. The FCS apparatus comprises an
5 inverted fluorescence microscope 1 using a confocal
optical system, a photo-detector such as a photo-
multiplier 2 for measuring fluorescence from a test
sample, a data processing apparatus 3 for receiving
measurement data from the photo-multiplier 2 and
10 performing arithmetic operation by applying an
autocorrelation function, thereby indicating the
measurement data numerically or graphically, and a
display apparatus 4 for displaying the result of the
arithmetic operation on a screen.

15 A test sample fluid 11 can be simply set on
a slide glass 13 mounted on a sample holder 12 by
attaching the fluid thereon dropwise, as shown in
FIG. 1. In this apparatus, since the test sample fluid
11 is used in an extremely small amount, a cover 14
20 is placed on a slide glass 13 in order to prevent
vaporization of a moisture content of the fluid. It is
preferred that the cover 14 is formed of a material
with a minimum light transmissivity, since airtightness
and light shielding properties can be simultaneously
25 obtained. However, the inner surface of the cover may
be preferably formed of a material with a minimum light
reflectance in order to prevent reflection of an

excitation light beam. Immediately under the portion of the slide glass 13 on which the test sample fluid 11 is placed, an objet lens 15 is mounted in such a way that the focus is achieved in the test sample fluid 11.

5 Note that the fluorescence microscope 1 used herein may be a reflection-type fluorescent microscope. In the reflection-type fluorescent microscope, the test sample-containing fluid 11 may be directly attached dropwise on the lower surface of the objective lens 15.

10 In FIG. 1, argon (Ar) ion laser is used as the laser generating apparatus 16 which serves as a light source for the fluorescent microscopy 1. However, the light source may be varied depending upon a type of fluorescence. More specifically, a krypton-argon

15 (Kr-Ar) ion laser, helium-neon(He-Ne) laser, and helium-cadmium (He-Cd) laser may be used. In addition, various operations including loading/unloading of the slide glass 13 into/from the fluorescence microscope 1, dropwise attachment of the test sample fluid on the

20 slide glass 13, and open/shut operation of the cover 14, may be performed automatically, if necessary.

FIG. 2A is an enlarged view showing a measurement portion of the fluorescence microscope 1. In FIG. 2A, a micro detection field 20 is formed by appropriately

25 arranging positions of the slide glass 13 and an objective lens 15 having a predetermined numerical aperture (NA=1.2 in the figure). Actually, as shown

in FIG. 2, the micro detection field 20 forms nearly a cylindrical region having a certain volume, which extends up and down from a focal point of the laser beams (a tightened middle portion). The detection field 20 is defined by a length Z along the optical axis and an average radius W from a focal point (reference point). If the micro detection field 20 is reduced to a minimum level for monitoring the micro-motion of the fluorescent molecules, it is possible to measure individual fluorescent molecules accurately since noise derived from the fluorescent molecule (in the test sample fluid 11) not present near the focal point can be efficiently eliminated.

A third aspect of the present invention relates to a similar analytical method to the first aspect except that the marker molecule is conjugated to the primer for PCR rather than substrate molecule.

According to the third aspect, there is provided a method of quantitatively analyzing a target nucleic acid molecule present in a biological sample, comprising:

an amplifying step of amplifying the target nucleic acid by using first and second primer molecules having sequences which are complementary with two discrete nucleotide sequence regions of the target nucleic acid molecule respectively, at least one of the first and second primers being labeled with

a detectable marker molecule and at least the number of labeled primer molecules being known;

5 a measurement step of obtaining measurement data regarding the labeled molecule by using at least a part of a test solution which has been subjected at least a single amplification step; and

a determination step of determining a number and a size of the target nucleic acid molecules on the basis of the measurement data.

10 In the third aspect, a mixing ratio of the first primer and the second primer is not equal. In this respect, reference can be made to an asymmetric PCR method disclosed by Gyllenaten, U. B. et al. (Proc. Natl. Acad. Sci. USA 85, 7652-7656, 1988). However,
15 the asymmetric PCR method disclosed by Gyllenaten, U. B. et al., is directed to production of a single stranded DNA (ssDNA) as a PCR product in order to simplify the determination of the nucleotide sequence. Therefore, in the asymmetric PCR, if the PCR is performed by using
20 two types of PCR primers in an asymmetric ratio (that is, they are not contained in an equi-molar amount), after the minor primer is completely consumed during the PCR cycle, only ssDNA is produced in every following PCR cycle. The present invention is unique
25 in that the asymmetric PCR method is modified in order not to determine the nucleotide sequence but to quantitatively determine the target nucleic acid.

More specifically, in the present invention, a nucleic acid amplification reaction is performed with binding a marker molecule to the minor primer, and measuring a signal derived from the marker molecule bounded to the minor primer. Therefore, even when hybridization probabilities of the minor and major primers to the target nucleic acid are not equal, it is possible to securely and completely eliminate free marker molecules without performing the Bound/Free separation such as centrifugal separation or gel electrophoresis. As a result, the S/N ratio becomes extremely high. BY virtue of the high S/N ratio, the present invention brings an epoch making effect of attaining quantitative analysis of a nucleic acid with a high detection sensitivity. Accordingly, the method of the present invention also makes it possible to detect the target nucleic acid molecule during or prior to the PCR amplification with a high accuracy.

In the unique asymmetric PCR performed in the present invention, it is important that the amount of the second primer is set smaller than that of the first primer and that the second primer is labeled with an optically detectable marker molecule. As to the numbers of first and second primer molecules, it is preferable that the lowermost values thereof be set such that at least one cycle of the amplification cycle is effected under the presence of an excessive amount

of the labeled primer molecules. Furthermore, as shown in a conceptional diagram (FIG. 3) of the unique asymmetric PCR according to the present invention, the number (or amount) of the second primer molecules which are labeled with markers is a critical factor to determine necessary repeats of the hybridization cycle in the unique asymmetric PCR. FIG. 3 shows the case where five second primer molecules are mixed with an excessive amount of the first primers, per target nucleic acid molecule. The amplification reaction step of this case is preferably performed in a certain mixing ratio of the first and second primer molecules, which is selected so as to attain the asymmetric nucleic acid amplification. In particular, it is further preferable that one of the primers be contained in a smaller moles than that of the other primer, and that the minor primer be labeled with the marker molecule. The mixing ratio of two types of primers contained in the test fluid preferably falls within the range of 2:1 to 20:1, since the production efficiency in the amplification reaction becomes high within this range. Furthermore, a mixing ratio in concentration of two types of primers in the test fluid preferably falls within the range of 800 nM :400 nM to 800 nM :40 nM, since the yield of the PCR product becomes high within this range.

In the amplification reaction applying the unique

asymmetric PCR of the present invention, it is preferable that even if a test sample contains a large amount of the target nucleic acid, at least one cycle of the amplification reaction can be carried out completely without causing shortage of second primer. In the method of the present invention, it is possible to detect the target nucleic acid even when the target nucleic acid is present in a small amount. Therefore, the second primer can be further reduced if the test sample containing fluid is reduced. The number (or amount) of the second primer molecules may be statistically determined such that one or more PCR cycles are completely effected even when the target nucleic acid is present at the uppermost amount which may naturally occurring in a test sample subjected to PCR. Hence, in case that the amplification reactions are performed using the target nucleic acid molecules in various amounts and the second primer in a constant amount, when the smaller amount of the target nucleic acid molecule is used, the more cycles must be repeated until they reach the satisfactory level of amplification, i.e., until the second primer is completely consumed. Therefore, it is preferred that the amount of the second primer is set such that the second primer is consumed in an appropriate PCR cycles even if only one target nucleic acid molecule is present in the test sample subjected to the measurement. As is apparent

from FIG. 3, the amount of the second primer and the amount of the target nucleic acid contained in the test sample fluid have an influence on the time or number of PCR cycles required until the asymmetric PCR amplification reaction reaches a plateau or saturated. In the present invention, it is supposed that when the amount of either the first or second primer is set at relatively and significantly lower, the number of the primer molecules thus set lower should be known. If the known number of the primer molecules is small, both primers may be labeled with the same marker molecule. Accordingly, at least one of the first and second primers may be labeled in this case. On the other hand, since the major primer does not directly contribute to the measurement of the present invention, it is principally unnecessary to know the number of the major primer molecules.

After completion of effective cycles of PCR, at least a part of the test sample fluid is taken as an aliquot and subjected to measurement appropriately. Alternatively, the measurement may be carried out on the test fluid mixture contained in a PCR reaction container or the like. Any measuring method may be used as long as it can measure a change in size of the labeled molecule in the course of amplification. For example, gel electrophoresis, capillary electrophoresis, flow-cytometer for fluorescence measurement, image

cytometer, and fluorescence microscopy may be effectively used. If the measurement step is performed in a three dimensional micro detection field, free motion of the labeled molecule in the test sample fluid can be accurately determined while keeping its natural state, in accordance with the results of PCR reaction. The micro detection field can be formed by converging light from a halogen lamp or by passing the light through an aperture (pinhole or monomode fiber end) having an extremely small average radius. However, in this case, converged laser light is preferably used. More particularly, it is preferred to use an optical system in which an objective lens with an appropriate magnification and numerical aperture is employed so that the focal point is formed on an appropriate position within the test fluid.

If the micro detection field of the measurement step is formed by a confocal optical system, measurement data having a deep depth of field can be obtained. As a result, any individual marker molecules are always brought into focus in the detection field, and the accurate position of the marker molecules and output data of fluorescence can be supplied to the measurement means.

If the micro detection field falls within a diffraction limited region near a focus, the individual marker molecules can be measured with a high S/N ratio.

When the diffraction limited region is formed by a pin hole having an average diameter of $15 \pm 5 \mu\text{m}$, measurement data can be efficiently obtained from a small number of selected marker molecules.

5 When the micro detection field is a nearly cylindrical area having an average radius of $200 \pm 50 \text{ nm}$ and an average length on the optical axis of $2000 \pm 500 \text{ nm}$, it is possible to efficiently capture the free micro-motion of the marker molecule sighted in
10 the measurement detection field.

 In the present invention, mobility of the marker molecules moving in and out of the micro detection field is measured by using increase/decrease or appearance/disappearance of the intensity of signal
15 output from each marker molecule present in a predetermined space. Accordingly, the type of the marker molecule to be tagged to the second primer molecule is preferably capable of outputting a constant signal at a plurality of measurement time points. As the
20 material to be used as the marker molecule, there are a luminescent material, fluorescent material, magnetic material, radioactive material and the like. In particular, as the luminescent material and fluorescent material, a dye that emits luminescence or
25 fluorescence for a long time may be preferably selected. If the marker molecule having a luminescent dye or a fluorescent dye is used, measurement can be performed

at a molecular level by using an apparatus having
an optically simple structure. As a preferable
fluorescent dye capable of emitting a detectable signal
before and after the hybridization, there are FITC,
5 rhodamine, DAPI, Cy 3, Cy 3.5, Cy 5, Cy 5.5, Cy 7 and
the like.

When a positional change of the fluorescence
molecule is measured in the measurement step,
fluorescent signal can be received by a fluorescence
10 measurement means such as photo-multiplier or photo-
diode. The fluorescence measurement means that has
a measurement mode capable of measuring a single
photon, is useful for measuring individual fluorescent
molecules.

15 When fluctuation motion of the marker molecules
in a fluid is measured in the measurement step, the
present invention will be a more effective embodiment
since the micro-motion of each of marker molecules is
accurately measured. In the case where the fluctuation
20 motion is measured, arithmetic operation can be
preferably performed by applying autocorrelation
function. In particular, when fluorescent dye is
used as the marker molecule, it is preferred that
fluorescence correlation spectroscopy (simply referred
25 to as "FCS") be used. The FCS measurement data of
a biological material may be arithmetically operated
with reference to a report made by Kinjo et al. in

which FCS is used in a hybridization reaction between a labeled nucleic acid probe and a target nucleic acid molecule (Kinjo, M., Rigler, R., Nucleic Acids Research 23, 1795-1799, 1995).

5 Present inventor found that quantitative measurement of PCR is possible by using FCS in his own investigation. In his investigation, PCR was performed by using two types of primers in an equal amount, and by conjugating a fluorescent marker molecule to dUTP
10 which is one of nucleotide monomers used in PCR amplification. As a result, the target nucleic acid molecule was able to be detected even if PCR was repeated in a small number of cycles. However, in the method thus found, it was necessary to perform a step
15 for removing the fluorescent molecule not incorporated into the amplification product before the fluorescence measurement. Then, the present inventor further investigated and found a new method capable of quantifying the target nucleic acid molecule by PCR
20 without removing the unbound marker molecule.

An apparatus for performing FCS analysis is as explained above in connection with FIGS. 1, 2A and 2B, so that the explanation thereof will be omitted herein.

25 Based on the aforementioned various embodiment, the present invention is applicable to diagnosis for genetic disease, parent-child identification, criminal examination, gene treatment, molecular biology research,

development of medicinal products, and the like.

It should be appreciated that the aforementioned embodiment may altered and modified without departing the scope of the present invention. For example, in
5 a measurement apparatus explained in the aforementioned embodiments, a test sample is placed on a holder and measured under the fluorescence microscope in the same manner as the microscope observation. However, if an apparatus disclosed in European Patent Publication
10 No. 640828A1 is optically improved in such a way that the laser light emitted through an objective lens is focused on the test sample fluid within a thermal cycler, the amplification reaction can be continuously evaluated without transferring the test sample fluid
15 onto a slide glass. A plurality of data may be obtained in the course of the PCR cycle, either continuously or intermittently in a predetermined time after completion of a predetermine cycle. Alternatively, an appropriate number of measurement data may
20 be obtained in every different cycle.

Now, the present invention will be illustrated by way of examples, but the examples should not be construed any way to limit the present invention. The present invention may be modified in various ways
25 on the basis of the gist of the present invention and within the scope obvious from the state of the art at the time the present invention was filed.

Example 1

(1) PCR under the presence of fluorescence-labeled nucleotide

As a test solution, a mixture solution was
5 prepared which contained $0.1 \text{ U}\mu\text{L}^{-1}$ of Pol I type DNA
synthase (AmliTaq Gold, Perkin-Elmer), 1 X buffer I
(Perkin-Elmer), $200 \mu\text{M}$ of dATP, dGTP and dCTP,
and $120 \mu\text{M}$ of dTTP, $80 \mu\text{M}$ of fluorescein 11-dUTP
(FluoroGreen, Amersham Flu-dUTP), and $0.8 \mu\text{M}$ of
10 a forward primer and a reverse primer. Then, using
 $5 \mu\text{L}$ and $25 \mu\text{L}$ of the test solution, a standard
amplification of a nucleic acid was performed.

As a template, a lambda phage DNA having a total
length of 48.5 kb was used in an amount of $200 \text{ pg}/5 \mu\text{L}$.
15 A length of a target DNA was 4000 bp. The nucleotide
sequence of the primers used in the nucleic acid
amplification were as follows:

Forward primer: GATGAGTTCGTGTCCGTACAACT

(nucleotide number = 7131-7153)

20 Reverse primer: CTTAACCAGTGCGCTGAGTGACT

(nucleotide number = 11098-11120)

As a PCR apparatus for performing the nucleic acid
amplification reaction, a programmable thermal control
system (PC700, Astec Co. Ltd.) was used. As a PCR
25 container storing each aforementioned test fluid,
an oil-free reaction tube (Takara Shuzo Co., Ltd.)
was used. First, a denaturation treatment (initial

denaturation treatment) was performed at 96°C for 15 minutes. Thereafter, predetermined PCR cycles each consisting of an annealing treatment (incubation at 55°C for 15 minutes), an elongation reaction (incubation at 65°C for 4 minutes) and a denaturation treatment (incubation at 96°C for 1.5 minutes), were performed. After completion of the predetermined PCR cycles, the PCR container was removed from the PCR apparatus and stored at -20°C until next use.

To eliminate nucleotides not incorporated into the amplified product, distilled water was added to each of test solutions after the PCR reaction, to a final volume of 50 μ L, and each of the resultant mixture solutions was subjected to a MicroSpin column (S-400HR, Pharmacia Biotech Co., Ltd.). The volume Vc of each of the solutions collected by the column was measured based on the weight thereof, the volume Vc ranged from 55 μ L to 60 μ L. The solutions were further subjected to FCS measurement without purification. Assuming that a molecular weight of a single base pair was 660 Da, a molecular weight of the lambda phage DNA became 32×10^6 Da by calculation.

(2) FCS measurement

Using the FCS apparatus shown in FIG. 1, FCS measurement was performed. A part of the test solution previously subjected to the nucleic acid amplification was used as a test sample. The test sample was

attached dropwise on a sample slide glass. The sample slide glass was mounted on a sample holder of a commercially available FCS apparatus (ConfoCor, CarlZiss Jena GmbH). The test sample was excited by
5 CW Ar⁺ laser beam passed through an objective lens (C-Apochromat, NA=1.2) of a 40 X magnification.

An emitted fluorescence light was measured by using Avalanche photo Diode (APD) (SPCM-200-PQ, EG&G) in a single photon counting mode. The fluorescence signal
10 thus measured was analyzed and evaluated by a digital correlator (ALV5000/E (ALV GmbH)). It should be noted that the test sample actually subjected to the measurement was attached dropwise onto a cover glass in an amount of 20 μ L and a small box was placed on
15 the sample holder so as to cover the cover glass for preventing vaporization during the measurement.

The sample volume at the region near a focal point was determined by using a diffusion count of rhodamine 6G. The volume element was determined by using concentra-
20 tions of the fluorescein and Flu-dUTP solution.

The fluorescence signal was measured in every about one minute. Analytical evaluation was performed by programming so that the obtained fluorescence signals were sequentially stored in a memory section
25 and applied to a fluorescence autocorrelation function $G(t)$. The value of fluorescence autocorrelation function $G(t)$ was calculated from an average number N

of fluorescence molecules within a detection region,
translational time τ_{mono} of a labeled free substrate
molecule (Flu-11-dUTP), namely a nucleotide monomer
(monomer DNA), and translational time τ_{poly} of
5 a labeled substrate molecule incorporated into the
amplification product, namely a polymer DNA, by use
of the following Equation 1 based on a method disclosed
by Rigler et al. (see Fluorescence Spectroscopy-New
Methods and Applications, Springer, Berlin 13-24, In J.
10 R. Lakowicz (Ed.), 1992):

$$G(t) = 1 + \frac{1}{N} \left[\left\{ \frac{1-y}{1 + \frac{t}{\tau_{\text{free}}}} \sqrt{\frac{1}{1 + s^2 \cdot \frac{t}{\tau_{\text{free}}}}} \right\} + \right. \\ \left. \left\{ \frac{y}{1 + \frac{t}{\tau_{\text{poly}}}} \sqrt{\frac{1}{1 + s^2 \cdot \frac{t}{\tau_{\text{poly}}}}} \right\} \right]$$

... Equation 1

In Equation 1, y is a ratio of the polymer DNA;
15 $\tau_{\text{mono}} = W_o^2/4D_{\text{mono}}$, $\tau_{\text{poly}} = W_o^2/4D_{\text{poly}}$, $S=W_o/Z_o$,
wherein, W_o is a diameter of a volume element of nearly
a cylindrical measurement region formed in the micro
detection field (see, FIG. 2B), and $2Z_o$ is a length of
the volume element. D_{mono} and D_{poly} are translational
20 diffusion coefficients of the monomer DNA and the
polymer DNA, respectively.

Data analysis was performed by applying a non-
linear least square parameter method for calculating

5 $T = (N/V_o)V_c$... Equation 2

wherein V_o is a volume element and V_c is a volume of a solution collected by a spin column. During the FCS measurement, an average fluorescence intensity was also measured. The intensity reflects the total number of fluorophores (Flu-dUTP) incorporated into the DNA amplified in the volume element. The value obtained by dividing a count rate of an average fluorescence intensity by the number of the DNA molecules (N_y) was employed as an average count rate (C/M) of the DNA molecules. The value was able to be used in practice as an effective marker concentration of the PCR products.

The volume element of the measurement region was determined by measuring diffusion times of reference samples such as rhodamine 6G, fluorescein, and Flu-dUTP. A ratio of length to diameter of the measurement region (see FIG. 2B) formed near a focal point was obtained from the value of S in Equation 1. The ratio was about 0.2. The volume element V_0 was obtained as 1×10^{-15} L through calculation.

(4) Incorporation of Flu-11-dUTP into PCR products

In order to elucidate a functional effect of fluorescence-labeled nucleotide in PCR, a concentration of Flu-dUTP was changed from 10% to 50%. FIGS. 4A and 4B are electrophoretic (agarose gel) photographs showing characteristics of amplified 4 kb products. More specifically, 3 μ L of a PCR reaction mixture obtained after 50 cycles of the nucleic acid amplification was subjected to electrophoresis on 7% agarose gel. FIG. 4A is a photograph before staining with ethidium bromide, whereas FIG. 4B is a photograph after staining with ethidium bromide. In the figures, M denotes a molecular marker (Hae III-digested ϕ X 174 DNA). In the figure, numerals "0", "10", "20", "30", "40" and "50" indicate a percent ratio of dUTP to dUTP+dTTP. A symbol "FN" denotes a free Fluorescent Nucleotide (not incorporated into PCR product). When the fluorescence emitted from the gel was measured, an orange-colored optical filter Ya3 was used in FIG. 4A and a red-colored optical filter R1 was used in FIG. 4B.

According to FIG. 4A, a fluorescent PCR product of 4000 bp was able to be synthesized under the presence of 50% Flu-dUTP. The fluorescent intensity of the labeled DNA increased with the increase of concentration of Flu-dUTP. The total production amount of the amplified DNA can be exhibited by ethidium staining. This experiment demonstrated that the total production amount is the same in the case where no Flu-dUTP was

contained.

(5) Fluorescence intensity and amplification
product amount in PCR

FIG. 5 is a graph showing the relationship between
the marker concentration and the product amount in PCR,
which was obtained as a function of a percent ratio of
Flu-dUTP. In the figure, a square mark indicates an
average count per DNA molecule (C/M). A solid (black)
mark indicates the number of DNA molecules present
in the measurement region of an detection field.
In FIG. 5, it is clearly shown that the fluorescent
intensity per molecule (C/M) increases with an increase
of Flu-dUTP. This result demonstrates that the
marker concentration depends upon the concentration
of Flu-dUTP. In addition, the number of DNA molecules
is almost constant within the range of Flu-dUTP
concentration used in the experiment. This result
is consistent with electrophoretic analyses shown
in FIGS. 4A and 4B. This fact demonstrates that
fluorescein does not prevent the activity of DNA
synthase. When C/M values of free Flu-dUTP (monomer)
and the PCR product are compared, the number of
Flu-dUTP monomers incorporated into a 4000 bp DNA chain
is obtained. It shows the possibility that modified
nucleotides are present adjacent to each other.

(6) PCR monitor by FCS

FIG. 6 is a graph showing a change of the value of

autocorrelation function $G(t)$ with time (t) during the PCR reaction, and more specifically, showing a typical change of the correlation curve with time. In the figure, a solid circular symbol, triangular symbol, reverse-triangular symbol, diamond-shaped symbol, and square symbol respectively indicate values measured at the time 10, 20, 30, 40 and 50 cycles of the nucleic acid amplification are completed. As is apparent from FIG. 6, the value (plot on the Y-axis) of the autocorrelation function at zero time-lag decreases with the number of PCR cycles. The results are due to an increase in number of DNA molecules.

FIG. 7 is a graph obtained by normalizing each of the autocorrelation functions at zero time-lag. In FIG. 7, a curve of Flu-dUTP is indicated by solid circles for comparison. In the figure, a solid line, a broken line, a dotted line, a dash-dot line, and a dashed-dot-dot line respectively indicate values measured at the time 10, 20, 30, 40 and 50 cycles of the nucleic acid amplification are completed. As is apparent from FIG. 7, the autocorrelation (solid line) obtained after 10 cycles is distinct from that of Flu-dUTP. This autocorrelation was not able to be applied to a simple one-component model, but applied to a two-component model as shown in the aforementioned Equation 1. Even in the test solution obtained after 50 cycles of PCR were completed, a short diffusion-time

component was detected. The short diffusion-time component is defined as non-reacted Flu-dUTP which passed through a spin column.

FIG. 8 is a graph showing the number of amplified DNA molecules in a PCR reaction solution (5 μ L) versus the PCR cycle number. The total number of amplified DNA molecules was calculated by the aforementioned Equation 2. The experiment was performed by varying an initial amount of a lambda phage DNA. In the figure, a square symbol shows the case where the initial amount of the lambda phage DNA is 200 pg (3.75×10^6 copies). A circle symbol, triangular symbol, and inverted triangular symbol respectively show the cases that the initial amounts are 20 pg (3.75×10^5 copies), 2 pg (3.75×10^4 copies), and 0.2 pg (3.75×10^3 copies), respectively.

FIG. 9 is a graph showing the relationship between the number of DNA molecules after 20 cycles and the initial number of templates. When a DNA is amplified by PCR, the DNA molecules exponentially increases before reaching a plateau. Since the template molecules is amplified to 1024 (2^{10}) times of the initial number, the DNA of 3.75×10^6 copies (corresponding to 200 pg) results in 3.75×10^9 copies. However, when the initial copy number is 3.75×10^6 , 7.44×10^9 copies are determined by FCS measurement after 10 cycles. Similarly, when the initial copy

number is 3.75×10^5 , 0.31×10^9 copies are respectively determined after 10 cycles. Thus, the quantitative performance of FCS determination seems low in faithfulness (accuracy) in the early state of the test sample amplification. This is because that the number of amplified DNA is low in the detection field and many non-incorporated free labeled nucleotides are present.

In such a PCR quantification by FCS on the basis of direct fluorescence labeling, it is possible to estimate an initial concentration of the target DNA at a level of 10^3 , even after 10-cycle amplification. As is apparent from FIG. 9, however, the initial concentration of the target DNA can be determined more clearly after 20 cycles are completed. As shown in this figure, since there is a linear relationship between the initial number of DNA molecules and the number of the amplified DNA molecules, it is possible to quantify the target DNA molecule. Although the data is omitted, it was confirmed that the quantification could be made by applying another calibration curve and another DNA sequence of 500 bp. Furthermore, 20 cycles of PCR were performed by using 5 μ L of test sample solution in various experiments. If the amount of the test sample solution is increased, it goes without saying that a high reproducibility can be obtained more accurately. This is the most important feature to

apply this detection technique to diagnosis. It is therefore preferable that an appropriate cycle number be selected through experiments to provide a good reproducibility for saving time and materials.

5 As is shown in the aforementioned experiments, the present invention succeeded for the first time in applying FCS to a molecule-counting method for use in PCR with direct labeling. Furthermore, it was proven that PCR is not inhibited even if 50% of dTTP is
10 replaced with Flu-dUTP in the case where a 4000 bp target nucleic acid is amplified. When shorter target nucleic acids, for example, 500 bp, 1000 bp, and 2000 bp are used, a more intensive fluorescence band was detected by electrophoretic analysis after 50
15 thermal cycles. Since a detectable marker molecule such as fluorescent molecule is bound to a substrate molecule which is used in a general PCR, this method can be easily employed in a general laboratory in the same way as in a general PCR process. The FCS assay
20 described herein only requires slightly modifying the general PCR conditions, using a small amount (5 μ L) of reaction solution as a test fluid, and performing a small-number of PCR cycles. The fluorescent marker molecule to be incorporated into a DNA molecule can be
25 conjugated with any types of gene markers and infection markers. Therefore, the method of the present invention can be applied to such nucleic acids derived

from various creatures by utilizing the previously established know-how of the PCR method.

The quantitative PCR based on FCS according to the present invention is useful in various fields because of the following reasons. First, this method has a high sensitivity, saves time and labor, and is performed by employing a reaction solution of extremely simple composition and a simple operation process.

Second, the FCS measurement is performed non-invasively.

Third, the test fluid after FCS measurement is completed may be used conveniently in the following experiments such as fluorescence hybridization and FISH analysis. Furthermore, a digestion reaction process with a restriction enzyme performed in a homogeneous solution can be measured by using FCS. Therefore, a point mutation and RELF etc. can be analyzed by using FCS with highly labeled DNA as described above, continually after amplification without using a isolation procedure such as column chromatography and gel chromatography. These PCR quantification methods based on FCS are applied to diagnosis and screening experiments, and further, can develop a new field for detecting molecules at a single molecular level.

As described herein, two important parameters in biological science can be obtained by using FCS. These parameters are an average number of molecules and a translational diffusion coefficient of the molecules

within a detection region (10^{-15} L). In the present invention, PCR products were analyzed at a single molecule level by using FCS. A specific DNA nucleotide sequence can be quantified before the PCR amplification reaches a plateau. In order to apply FCS to PCR, PCR is performed in the presence of the nucleotide monomer labeled with fluorescein 11-dUTP, which is used as a substrate in an enzymatic amplification cycle.

Through the PCR reaction mentioned above, a target DNA of 4000 bp were labeled. After 10 cycles, an amplified DNA product is detected by increased diffusion time of the fluorescent molecules in the FCS detection region. The initial number of target DNA molecules in a micro-volume ($5 \mu\text{L}$) of the detection region ranged from 3.75×10^6 to 3.75×10^3 copies.

As described in the foregoing, the PCR amplification reaction in this Example is performed by using a substrate molecule labeled with a marker molecule. In addition, mobility of the marker molecules is measured. As a result, an amount of the target nucleic acids present in a test sample can be analyzed quantitatively and accurately in a simple process.

Example 2

Amplified amount of 500 bp DNA by Rho-primer and the total number of fluorescent molecules arithmetically calculated by using FCS measurement

This Example relates to fluorescence-labeled

primer-based asymmetric PCR (referred to FA-PCR hereinafter).

(1) Implementation of unique asymmetric PCR

Preparation of primer and a mixture solution for

5 PCR

Asymmetric primer pairs were selected from two types of non-labeled primers, i.e., primer 1: 5'-GATGAGTTCGTGTCCGTACAACCTGG-3' and primer 2: 5'-GGTTATCGAAATCAGCCACAGCGC-3' (manufactured by Hokkaido System Science), and two types of primers, Rho-primer 1 and Rho-primer 2 (manufactured by Takara Shuzo Co., Ltd.) which are prepared by labeling the aforementioned primers 1, 2 with a fluorescent molecule Rhodamine in combinations and concentrations shown in Table 1. Each of the primer pair was mixed with a full length lambda phage DNA (48.5 kbp, 3.2×10^7 Da, 1 bp=660 Da) serving as a template, a buffer (25 μ L) containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, and 0.001% gelatin, pH8.3, together with 2.5U polymerase AmplitaqGold™ (manufactured by PE Applied Biosystems) and 0.2 mM of dNTP. The concentration of a template was varied between 1 ng/25 μ L and 0.1 fg/25 μ L which correspond to 1.9×10^7 to 9×10^1 molecule/25 μ L.

Implementation of PCR amplification reaction

25 The PCR reaction of a nucleotide sequence of a 500 bp template DNA was performed by using a programmable thermal controller, Model PC-700

(manufactured by Astech). First, pre-incubation was performed at 96°C for 15 minutes as a pre-denaturation step. Thereafter, a PCR cycle consisting of an annealing step at 55°C for one minute, an elongation step at 72°C for 2 minutes, and a denaturation step at 96°C for one minute was performed in 50 cycles.

(2) Measurement of amplification reaction results by using FCS

Measurement of a test sample mixture solution

After 50 cycles of the amplification reaction, an appropriate amount (10 μ L) of a test sample mixture solution was attached dropwise onto a cover-glass Lab-Tek (trade name, manufactured by Nalge Nunc) by pipetting. Then, the cover glass was mounted on a sample holder of an inverted FCS apparatus having a confocal optical system, ConfoCor (trade name, manufactured by Carl Zeiss). An objective lens C-Apocromat (trade name, Carl Zeiss) having a numerical aperture of 1.2 was focused on the test sample fluid on the cover-glass. In order to prevent vaporization of the test sample fluid during measurement (one minute) using argon laser excitation light, an additional similar cover-glass was placed to cover the droplet of the test-sample containing fluid attached on the cover-glass. Then, fluorescence was measured at room temperature. Incidentally, when the volume of detection region, which is a micro detection field of

the FCS apparatus, was defined by measuring the diffusion time using rhodamine 6G as a reference test sample. As a result, the ratio S of the diameter to the length of the detection region was about 5.2.

5 The volume element was 1×10^{-15} L according to arithmetic calculation. After the FCS measurement, the test sample mixture was subjected to agarose gel electrophoresis, thereby identifying fractions.

Conversion of measurement data to statistical data

10 The intensity distribution of the laser beam on a focal point was a Gaussian distribution. However, the fluorescence observation area was expressed by a nearly cylindrical shape as in FIG. 2B, and considered as such. The laser beam focused in test sample fluid forms
15 an extremely small (micro) cylindrical field where fluorescent particles are moved in and out by the Brownian motion. As a result, the fluctuation of fluorescence intensity caused by a change in number of fluorescent particles in the micro cylindrical field
20 can be monitored. The fluctuation of fluorescence intensity was analyzed by applying an autocorrelation function to obtain an average number of molecules within a focus region and translational diffusion time. The fluorescence autocorrelation function $G(t)$ is as
25 explained previously. The data analysis by FCS was performed by a computer program called "FCS ACCESS" (EVOTEC BioSystems Co., Ltd.) applying the non-linear

least square method.

Primer concentration			y ^a	N ^b	Total ^c
Primer 1 (nM) (Rho- primer1) ^d	Primer 2 (nM) (Rho- primer2) ^d	Primer ratio			
800	400 ^d	1:0.5	0.30	900	120
800	40 ^d	1:0.05	0.60	90	24
800	4 ^d	1:0.005	0.52	8	2.1
400 ^d	800	0.5:1	0.35	863	140
40 ^d	80	0.05:1	0.21	76	8.4
4 ^d	800	0.005:1	Nd ^e	9	Nd ^e

In fitting, to reduce free parameters, τ free (0.17 ms) and τ poly (1.98 ms) were fixed to reduce free parameters. In FIG. 1, a is a yield of elongated rhodamine-labeled primer, b is an average number of fluorescent molecules in the focussed field, c is a total yield calculated by using an initial concentration of a fluorescent primer and a yield of elongated rhodamine-labeled primer (y), d is a concentration of the rhodamine-labeled primer, and e indicates "not detected".

Table 1 is the results of a preliminary experiment performed in order to determine an optimum ratio of a primer set to be used in the asymmetric PCR. Six primer ratios were tested, 3 for primer 1:Rho-primer 2 (800:400 nM, 800:40 nM and 800:4 nM) and 3 for Rho-primer 1:primer 2 (400:800 nM, 40:800 nM and 4:800 nM) in the presence of 1 ng of template. After 50 cycles of PCR, 10 μ L of the reaction solution were set on the cover glass and then measured by FCS

for 1 min. FIG. 10 shows typical fluorescence auto-correlation functions. The translational diffusion time of Rho-primer 2 ($\tau_{\text{free}} = 0.164$ ms) was defined from a control sample that had not been subjected to a thermal cycle (solid circle in FIG. 10).

After 50 cycles of PCR, the autocorrelation function curve of a free Rho-primer (plotted by a circle in FIG. 10) and the autocorrelation function curve of an asymmetric PCR product were obtained by fitting data thereof in Equation 1. Then, the translational diffusion time of the free Rho-primer ($\tau_{\text{free}} = 0.164$ ms) and the translational diffusion time of an amplified product DNA ($\tau_{\text{poly}} = 1.98$ ms) and the yield ($y = 0.6$) thereof were given to Equation 1.

Another control sample, minus enzyme but with the thermal cycle, was also measured (data not shown), and the result was 0.169 ms. The translational diffusion time of 500-bp DNA (PCR products) was evaluated from curve fitting of autocorrelation data, giving $\tau_{\text{poly}} = 1.98$ ms (a curve plotted by a square in FIG. 10). The value agreed with the 500-bp length DNA according to theoretical calculation of the translational diffusion constant of rod-like molecules.

Therefore, the amplified product was well identified from the change in the autocorrelation function.

Due to the different translational diffusion times of the free primer and the amplified primer, it was able

to quantify the fractions of amplified products.

In the analysis, the values of τ_{poly} , τ_{free} and S were fixed in the fitting to reduce the number of free parameters and clearly separate the ratio of free-to-extended primers (y). Although the free primer and PCR products were not physically separated after the thermal cycle, it was possible to measure the fractions of products by this analysis (Table 1). In the case of primer 1:Rho-primer 2 is 800 nM :40 nM, the ratio between the remaining free primer and the PCR product ($y=0.6$) was better than with the others. At the primer ratio of 800:400 nM, the total product of 500 bp was better than at other primer ratios. However, approximately 70% of the free primer still remained. Although it was expected that all of Rho-primer 2 would be incorporated into the PCR product at the primer ratio of 800:4 nM, only about half of the labeled primer 2 ($Y = 0.52$) could be incorporated, so the yield of product was not better than at the ratio of 800:40 nM. Therefore, a preferable primer concentration ratio was either 800 nM :40 nM or 800 nM :40 nM. Hence, the primer concentration ratio may be selected from the range between the primer concentration ratios. In general, it is considered that a preferably primer ratio ranges from 2:1 to 20:1. Subsequently, the marker molecule was attached to the primer 1 conversely. When the PCR is performed by

using Rho-primer 1 and primer 2 in a ratio of
400:800 nM, 40:800 nM, and 4:800 nM, the yield of the
PCR product was 0.35 or less. Thus, it is preferable
to appropriately bind the marker molecule to either of
5 a pair of primers, depending upon a type of target
nucleic acid molecule and primer molecule as well as
a chain length thereof so as to increase the PCR yield.

FIGS. 11A and 11B show the electrophoretic
(agarose gel) results of an amplified target product of
10 500 bp obtained after quantitative determination by FCS
measurement. Asymmetric PCR solution after 50 cycles
(3 μ L) was applied to 1.4% agarose gel for characteri-
zation of asymmetric PCR products with different primer
ratios by agarose gel electrophoresis. FIG. 11A is a
15 case where no ethidium bromide staining was performed.
FIG. 11B is a case where ethidium bromide staining was
performed. Lane 1: ϕ X 174 marker digested with Hae
III; Lane 2: a ratio of primer 1:Rho-primer 2 is
800:400 nM; lane 3: primer 1:Rho-primer 2 is 800:40 nM;
20 lane 4: a ratio of primer 1:Rho-primer 2 is 800:4 nM;
lane 5: a ratio of Rho-primer 1:primer 2 is 400:800 nM;
lane 6: a ratio of Rho-primer 1:primer 2 is 40:800 nM;
lane 7: a ratio of Rho-primer 1:primer 2 is 4:800 nM;
lane 8: control (PCR is performed by using normal
25 primers; and lane 9: control (using Rho-primer 2), and
control (PCR is performed by using Rho-primer 1 and
Rho-primer 2). The locations of 500 bp and Rho-primer

2 are indicated by arrows. A faint and broad band between 500 bp and the Rho-primer is a bromophenol blue dye.

In all cases, the rhodamine fluorescent band of 500 bp was able to be detected without ethidium staining (FIG. 11A). The PCR products were also detected by ethidium staining performed after electrophoresis (FIG. 11B). ssDNA (single stranded DNA) of 500 bp was not clear in the gel because the fluorescent intensity of the ssDNA is less than that of dsDNA (double stranded DNA) in this staining method. In the cases of a ratio of Rho-primer 1: primer 2 is 40:800 nM and 4:800 nM, a smear and a non-specific band were detected in the gel (FIGS. 11A and 11B, lanes 5-7). Conversely, in the cases where a ratio of primer 1:Rho-primer 2 was 800:400 nM, 800:40 nM, and 800:4nM, the 500 bp band was clearly detected, and the intensity thereof decreased as the primer ratio changed from 1 :0.05 to 1 :0.005 (FIGS. 11A and 11B, lanes 2-4). These results exactly corresponded to FCS determination results (Table 1). The ratio of primer 1:Rho-primer 2(800:40 nM) was used in further experiments in this work because the yield of 500 bp($y = 0.6$) was better than with other ratios. FCS-based, FA-PCR detection would have the advantages of quantitative accuracy and simplicity. One can detect a target gene by 1-min FCS measurement with only 10 μ L of reaction solution after

FA-PCR amplification. It would be interesting if a low copy number of template genes could be amplified by FA-PCR and then detected by FCS.

FIG. 12 shows the results obtained by changing an initial template concentration of a reaction solution (25 μ L) prior to PCR from 1.9×10^7 to 1.9, in order to investigate the sensitivity of the aforementioned method of the present invention (an inverted open triangular symbol indicates 1.9×10^7 , a solid triangular symbol indicates 1.9×10^6 , an open circular symbol indicates 1.9×10^5 , a solid square symbol indicates 1.9×10^4 , an open diamond symbol indicates 1.9×10^3 , an open inverted triangular symbol indicates 1.0×10^2 , an open triangular symbol indicates 1.9×10^1 , and a solid circular symbol indicates 1.9). The autocorrelation function $G(t)$ of Rho-primer 2 shifted toward a longer correlation time (toward right hand side of FIG. 12) in proportion to an increase in template number after 50 thermal cycles. This shift corresponds to an increase of the elongated Rho-primer 2. The yield and the number of FA-PCR products were calculated by Equation 1.

An average number of fluorescent molecules in an optical field is shown in FIG. 13. No specific influence on the number of labeled DNA molecules was observed, so that specific or nonspecific adhesion or partitioning to the surface of the tube and/or glass

was not detected in this experiment. FIG. 14 is a graph showing a yield (%) of 500 bp versus an initial template concentration. The yield of the product was dependent on the initial number of template molecules from 10² to 10⁴ molecules/25 μ L, which can be used as the quantification range. Even in the initial presence of 190 template molecules in 25 μ L, the correlation decay of amplified DNA was separated, and it could be detected clearly. A plateau effect was observed with over 10⁵ template molecules after 50 cycles. Although a number of factors could have contributed to the plateau effect, it was simply related to the number of cycles. Therefore, the quantification range will be optimized by reducing the number of thermal cycles for a higher concentration of initial template molecules.

The present inventors conducted experiments to set the PCR reaction conditions more optimum. As a result, even in the case where 20 of the template DNA molecules are present in a test sample, the detection of the template DNA molecules was successfully performed. If other conditions for PCR are more optimized, it is expected easy to design the detection method in such a way that 5 template DNA molecules or less can be detected. Such experimental results suggest that only one molecule of the template DNA may be principally detectable in the method of the present invention.

FIG. 15 shows the initial template number present

in a mixture solution of 25 μ L versus the yield (%) of the PCR product with respect to each of different PCR cycles. According to the results, it is demonstrated that asymmetric PCR of 20 cycle is sufficient to

5 quantify the target DNA in the case where the initial template molecules are present in as large number as 10^6 or more. Similarly, 30 cycles in the range of 10^4 to 10^6 , 40 cycles in the range of 10^2 to 10^4 , and 50 cycles or more in the range of 10^3 or less are

10 possibly used in the quantification of the target DNA. In particular, when the initial templates are present in a high concentration, the quantification available range can be optimized by reducing the PCR cycle number. If data is obtained within the quantification-ensuring

15 number of cycles as mentioned above, it is possible to determine the initial number of templates by, for example, multiplying the cycle number.

FIG. 16 is a graph showing the yield of a PCR product versus the PCR cycle number with respect to

20 the initiation number of the template DNA (data was originated from those of FIG. 15). From the results, it is demonstrated that if the templates are present in an initial number of 2×10^3 , the determination can be performed precisely. In consideration of the data of

25 FIG. 15, it is presumable to determine even if the templates are present in an initial number of 2×10^2 .

As described, in this example, the PCR

amplification reaction is performed by labeling a primer with a marker molecule. Furthermore, behavior of each marker molecule is statistically analyzed through the observation within a micro detection field.

5 It is therefore possible to accurately detect the amount of the target nucleic acid present in a test sample. In addition, since the marker molecule is bound to a second primer present relatively and significantly in a small amount, a free marker molecule
10 is consumed certainly and completely during the PCR cycles, with the result that the Bound/Free separation of the marker molecule is not required. Accordingly, this method is easily handled, applicable to automatic operation, and reduces reagent cost.

15 <Discussion for Examples 1 and 2>

Recently, some reports regarding a method of detecting a target gene by use of FCS have been provided. It has been found that Nucleic acid sequence-based amplification (NASBA) was combined with
20 FCS and has proven to be a useful detection method for human Immunodeficiency virus (HIV) diagnosis in plasma (see Oehlenschlaeger, F. et al, Proc. Natl. Acad. Sci. USA, 93, 12811-12816, 1996)). In the NASBA method which is one of nucleic acid amplification methods, two
25 primers and an additional fluorescence-labeled primer serving as a probe are required in the presence of three enzymes, namely T7RNA polymerase, reverse

transcriptase, and RNaseH. A simpler method has been reported by the same group, amplified probe extension (APEX) detection by FCS, but it still uses an extra fluorescence-labeled primer as a probe sequence besides a forward primer and a reverse primer set (Walter, N. G. et al., Proc. Natl. Acad. Sci. USA, 93, 12805-12810, 1996). A time shift of the autocorrelation function was observed after 26 cycles in that experiment.

A fluorescent quenching-based PCR quantification method, such as a fluorescent energy transfer analysis was previously reported (Heid, C. A. et al., Genome Res., 6, 986-994, 1996, Livak, K. J., PCR Method, Appl., 4, 357-362, 1995) and commercialized. This method employs two kinds of dyes in a single probe sequence other than a pair of PCR primers, as in the NASBA method and the APEX method described above. To design an oligonucleotide sequence of the probe, it is necessary not only to provide a nucleotide sequence that does not competing with the sequence of the primer but also to consider a melting temperature $T(m)$. In addition, the position between donor and acceptor dyes affects the efficiency of increase of fluorescent intensity and the 5' nuclease activity of polymerase.

Another fluctuation spectroscopy method (fluorescent cross correlation spectroscopy) has been reported as a detection method for a specific target gene in a homogeneous solution (Gastro. A. et al.,

Anal. Chem, 67, 3915-3920, 1997, Rudolf Rigler et al.,
J. Biotechnology, 63, 97-109, 1998). In the cross
correlation method, two probes or primers which bind to
different positions of the target nucleic acid, are
5 labeled with two types of fluorescent dyes having
different absorption wavelength. As a result, an
optical system of a measuring apparatus is inevitably
large and complicated for observing that primers
labeled with two types of fluorescent markers are
10 hybridized with a single nucleic acid molecule.
Although employment of the two types of dyes enables
to detect an extremely small amount of the target
nucleic acid sequence, this method still requires two
types of fluorescent probes (primers) and therefore
15 disadvantageous in view of convenience and reagent cost.

From the viewpoint of simplicity, the FCS-based
PCR quantification method offers a major advantage over
the energy-transfer and cross-correlation methods. The
fidelity and sensitivity of PCR are strongly dependent
20 on the type of enzyme (Pol I type or α type), selection
of primer sequence and reaction conditions. Once these
conditions can be fixed, FCS-based, FA-PCR can be
immediately applied to the ordinary PCR assay with
slight modification of the primer ratio. Therefore,
25 routine PCR conditions will be easily modified to
conditions for FA-PCR. Moreover, other amplification
methods, such as reverse transcription (RT)-PCR can be

modified for this method, and it should be possible to quantify the mRNA in single cells in the future.

In gel electrophoresis and column chromatography, the PCR product is separated from the primer and by-products. However, the target product is diluted during the electrophoresis and extraction procedures from the gel materials and then has a chance to come into contact with nuclease, leading to degradation. Conversely, the FCS method is convenient for sample collecting after measurement; a sample droplet is placed on a cover glass, and FCS is a noninvasive method, so the PCR solution is not diluted or destroyed during measurement. The FA-PCR detection method based on FCS described above, only the primer labeled with fluorescence under the reaction conditions is required without applying a physical separation method such as a gel filtration method. Although the resolution of gel electrophoresis is higher and can detect a single nucleotide difference in length, the very rapid analysis time (1 min in the Example) of FCS measurement compared with gel electrophoresis(1 h) will be advantageous for massive specimen analysis(for example, in diagnosis and screening). The screened sample from FCS can be immediately subjected to detailed analysis, such as gel electrophoresis, to confirm the FCS results or to analyze the nucleotide sequence. Moreover, the collected sample can be used in further experiments,

(for example, as a hybridization probe). If one need
not collect the amplified sample after FCS measurement,
it can be discarded without a risk of carryover
contamination because FCS is a non-contact measurement
method.

As FCS is noninvasive and allows direct measure-
ment, the PCR volume is dependent on the measurement
volume needed for FCS. Current measurements of FCS
were carried out with a 10- μ L sample volume, though the
sample volume can theoretically be reduced to a volume
element of the order of 10^{-15} L. However, the PCR
volume can be reduced to 1 μ L, and thus, it would have
a great advantage in comparison with other methods from
an economical point of view. Moreover, FCS is based on
fluorescence microscopy, and the detection field is set
on the objective, so that this method will be able to
quantify specific RNA and DNA from in situ PCR.

The present inventors used a two-component model
for analysis of the PCR solution and obtained good
results. However, many DNA species might be present in
other experiments. In such cases, species of DNA can
be well separated by conventional gel electrophoresis.
Although the gap in the correlation curve from the
model function to observed data of FCS could suggest
some other components distributed in the solution,
the simple two-component model seems to be the limit
in such cases. Consequently, when more detailed

quantitative analysis is necessary, multicomponent
FCS analysis(13) should be developed to obtain the
distribution function using a computer program such as
CONTIN(Max Planck Institute for Biophysical Chemistry,
5 Göttingen, Germany).

Without using a physical separation procedure,
this method is noninvasive, non-contact and uses a
small sample volume. Physical handling of such a small
sample volume can be done by using a capillary tube or
10 by using a small sample pit on a glass surface covered
by a thin cover glass or by film for on-line analysis.
With these properties and improvements, it is
straightforward to use, and the process used to monitor
amplification is simple(on-line monitoring) and can be
15 used with an automatic detection system (robotics).
The inventor of the present invention therefore
conclude that FCS-based, FAPCR quantification has
advantages in terms of sensitivity, quantitative
precision and simplicity. Since a spatial (three-
20 dimensional) resolution ability of the microscopy to
be used is the resolution of FCS, it is easy to measure
by spatially selecting a part of a cell. For example,
an intracellular molecular transportation of a fusion
protein can be measured by using GFP (green fluorescent
25 protein). Furthermore, it is expected that the method
of the present invention will be useful not only for
rapid screening, but also to open a new field of

CLAIMS

1. A method of analyzing a target nucleic acid by applying a nucleic acid amplification reaction to a test solution, said method comprises the steps of:

5 performing a nucleic acid amplification reaction of the target nucleic acid using a test solution containing a primer, substrate molecule at least one of which is labeled with a marker molecule capable of generating a detectable signal, a nucleic acid synthase,
10 and a target nucleic acid molecule;

measuring a signal from the marker molecule in the test solution after initiation of the nucleic acid amplification reaction;

15 evaluating mobility of the labeled molecule in the test solution on the basis of the signal detected; and quantifying the target nucleic acid molecule on the basis of evaluation results.

2. A method according to claim 1, wherein the measurement step includes a step of measuring an amount
20 of the marker molecule present in a predetermined measurement area.

3. A method according to claim 2, wherein, in the measurement step, a moving amount of the marker molecule within a predetermined time interval is
25 measured for a plurality of times.

4. A method according to claim 3, wherein the evaluation step includes a step of converting a change

of the moving amount into a statistical data, on the basis of a plurality of measurement data.

5 5. A method according to claim 4, wherein the conversion step includes a step of performing a arithmetic operation by means of an autocorrelation function.

6. A method according to any one of claims 1 to 5, wherein,

10 the method further comprises a step of removing the labeled substrate molecule not incorporated into the nucleic acid amplification products, between the step of performing the amplification reaction and the measurement step; and

15 the measurement step is performed in the state noncontact with the test solution.

20 7. A method according to any one of claims 1 to 5, wherein the quantification step include a step of determining the presence and absence of the marker molecule incorporated into the amplification products during the nucleic acid amplification, on the basis of the evaluation results.

25 8. A method according to any one of claims 1 to 5, wherein the quantification step includes a step of determining a number of the labeled nucleic acid molecules incorporated into the amplification products during the nucleic acid amplification on the basis of the evaluation results.

9. An apparatus of analyzing a target nucleic acid by applying a nucleic acid amplification reaction to a test solution, said apparatus comprises:

holding means for holding a test solution
5 containing a primer, substrate molecules at least one of which is labeled with a marker molecule capable of generating a detectable signal, a nucleic acid synthase, and a target nucleic acid;

measuring means for measuring a signal from the
10 marker molecule after initiation of a nucleic acid amplification reaction in the test solution;

evaluation means for evaluating mobility of the marker molecule in the test solution on the basis of the signal detected; and

15 data output means for outputting a evaluation result obtained by the evaluation means as a quantification data of the target nucleic acid molecule.

10. An apparatus according to claim 9, wherein the measuring means comprises an optical system for
20 performing measurement in a micro detection field brought within a diffraction-limited region near a focal point.

11. An apparatus according to claim 9, wherein the measuring means comprises a microscope for performing
25 measurement in a micro field of vision formed by a confocal optical system.

12. An apparatus according to claim 10 or 11,

wherein the diffraction-limited region is formed of an aperture having an average diameter of $30 \pm 20 \mu\text{m}$.

13. An apparatus according to claim 10 or 11, wherein the diffraction-limited region is formed of an aperture having an average diameter of $20 \pm 10 \mu\text{m}$.

14. An apparatus according to claim 10 or claim 11, wherein the micro detection field is a virtually a cylindrical region having an average radius of $200 \pm 50 \text{ nm}$ and an average length on an optical axis of $2000 \pm 500 \text{ nm}$.

15. An apparatus according to claim 9, wherein the evaluation means comprises a means for storing a plurality of measurement data obtained in a predetermined time, and an arithmetically operating means for processing the plurality of measurement data stored in accordance with an autocorrelation function.

16. An apparatus according to claim 15, wherein the evaluation means comprises a means for storing measurement data regarding to a plurality of marker molecules obtained in a measurement area, and an arithmetic operating means for processing the measurement data stored per each of the marker molecules in accordance with the autocorrelation function.

17. An apparatus according to claim 15 or 16, wherein the data output means includes a conversion means for converting the measurement data into statistical data expressing a positional change with

time with respect to a plurality of monitoring data.

18. A method of quantitatively analyzing a target nucleic acid molecule present in a biological sample, comprising:

5 an amplifying step of amplifying the target nucleic acid by using first and second primer molecules having sequences which are complementary with two discrete nucleotide sequence regions of the target nucleic acid molecule respectively, at least one of
10 the first and second primers being labeled with a detectable marker molecule and at least the number of labeled primer molecules being known;

 a measurement step of obtaining measurement data regarding the labeled molecule by using at least a part
15 of a test solution which has been subjected at least a single amplification step; and

 a determination step of determining a number and a size of the target nucleic acid molecules on the basis of the measurement data.

20 19. A method according to claim 18, wherein the amplification step is performed by using the first and second primers which are contained in a mix ratio selected to attain asymmetric nucleic acid amplification.

25 20. A method according to claim 18, wherein the number of one of the first and second primers is lower than that of the other primer, and the primer present

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in a lower number is labeled with a marker molecule.

21. A method according to claim 20, wherein the mix ratio of the first and second primers in the test solution falls within a range of 2:1 to 20:1.

5 22. A method according to claim 20, wherein a mix concentration ratio of the first and second primers in the test solution falls within a range of 800 nM :400 nM to 800 nM :40 nM.

10 23. A method according to claim 18, wherein the measurement step includes

a step of obtaining a plurality of measurement data within a predetermined time interval, in a micro detection field capable of identifying individual marker molecules;

15 a step of converting the plurality of measurement data into a statistical data showing a positional change with time; and

a step of determining a number of target nucleic acid molecules on the basis of the statistical data.

20 24. A method according to claim 23, further comprising a step of performing arithmetic operation of the statistical data by use of autocorrelation function.

25 25. A method according to claim 24, wherein a fluctuation motion of the marker molecule in the test solution is measured in the measurement step.

26. A method according to any one of claims 18 to 25, wherein the determination step is performed on the

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basis of a curve which is obtained by plotting the statistical data and shows a dynamic change of the target molecule.

27. A method according to claim 23, wherein the measurement step is performed in a three dimensional micro detection field.

28. A method according to claim 27, wherein the micro detection field in the measurement step is formed by a confocal optical system.

29. A method according to claim 28, wherein the micro detection field is a diffraction-limited region near a focal point.

30. A method according to claim 29, wherein the diffraction-limited region is formed by a pin hole having an average diameter of $30 \pm 20 \mu\text{m}$.

31. A method according to claim 29, wherein the diffraction-limited region is formed by a pin hole having an average diameter of $20 \pm 10 \mu\text{m}$.

32. A method according to any one of claims 26 to 31, wherein the micro detection field is virtually a cylindrical region having an average radius of $200 \pm 50 \text{ nm}$ and an average length on an optical axis of $2000 \pm 500 \text{ nm}$.

33. A method according to claim 18, wherein the marker molecule comprises a fluorescent dye.

34. A method according to claim 33, wherein the fluorescent dye generates a detectable signal both

before and after hybridization.

35. A method according to claim 34, wherein the fluorescent dye is selected from the group consisting of FITC, DAPI, rhodamine, Cy 3, Cy 3.5, Cy 5, Cy 5.5, and Cy 7.

36. A method according to claim 32 or 33, wherein the measurement step is performed by a measurement mode for measuring a single photon.

37. A method according to claim 18, wherein the
10 amplification step is performed in a number of cycles
which is determined depending upon an amount of the
target nucleic acid molecule.

ABSTRACT OF THE DISCLOSURE

5 A biological sample containing a target nucleic acid is mixed with a primer, a substrate labeled with fluorescence, and DNA polymerase to prepare a test solution. After a nucleic acid amplification reaction is performed in a predetermined manner, the test solution is attached dropwise on a slide glass, which is mounted on a sample holder of an inverted fluorescence microscope. Data measured by a photomultiplier are arithmetically operated by applying an auto-correlation function in a data processing apparatus. Based on the arithmetic results, quantification data for the target nucleic acid, are numerically or graphically displayed on a screen of a display apparatus.

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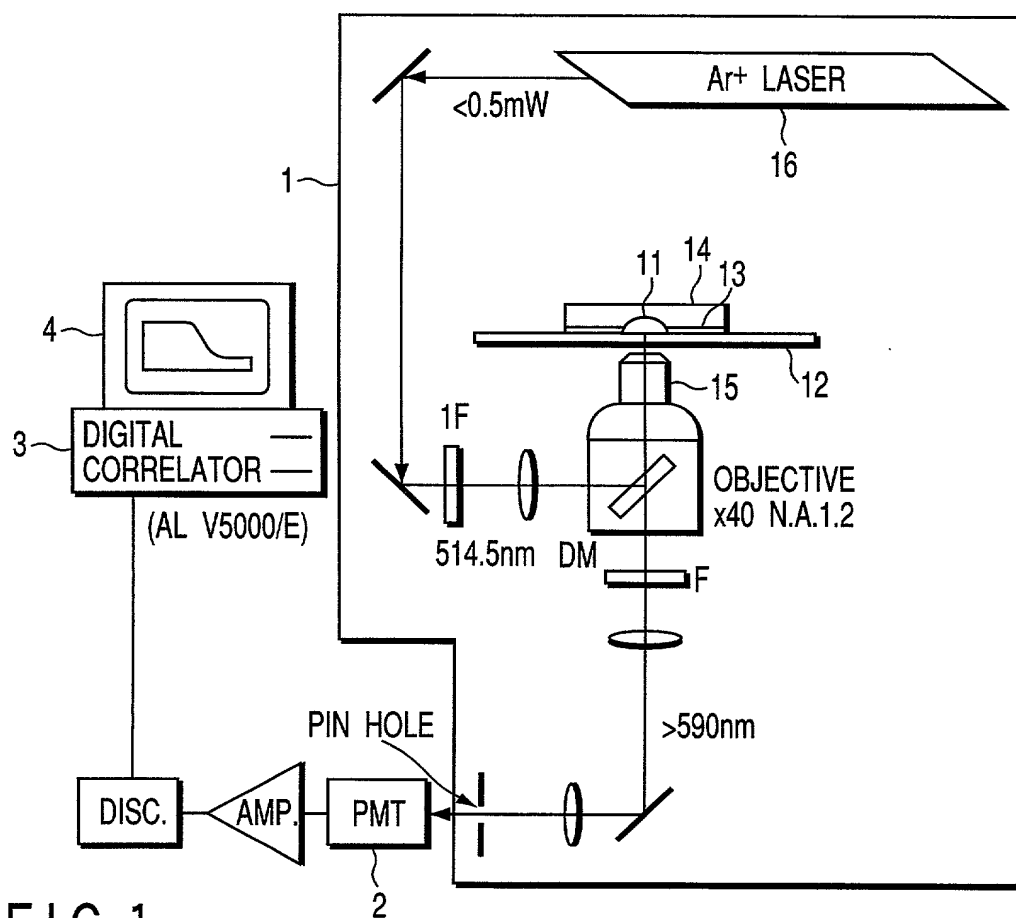


FIG. 1

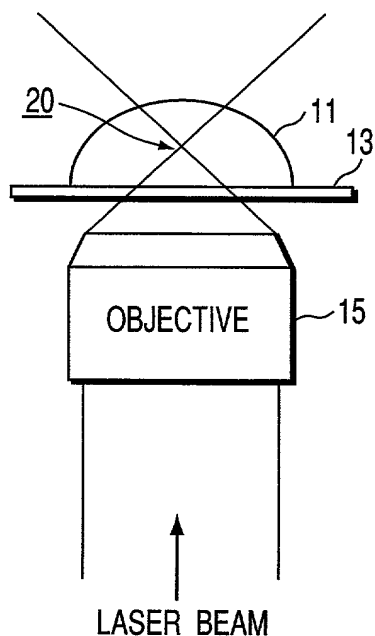


FIG. 2A

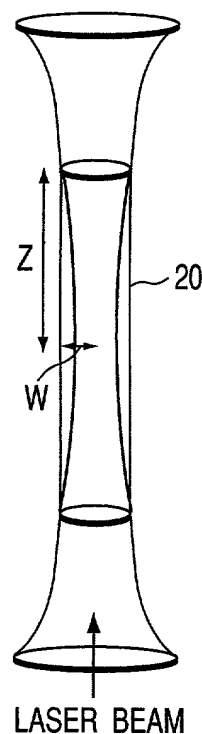


FIG. 2B

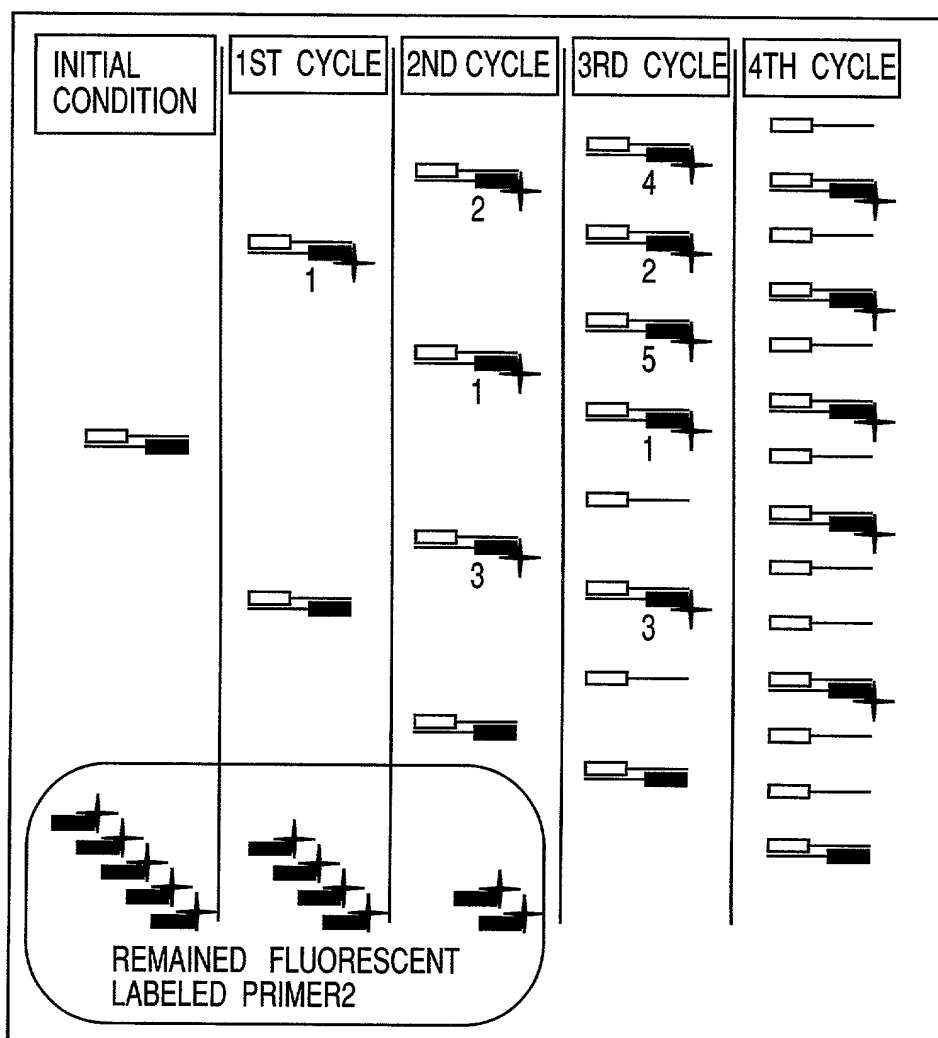


FIG. 3

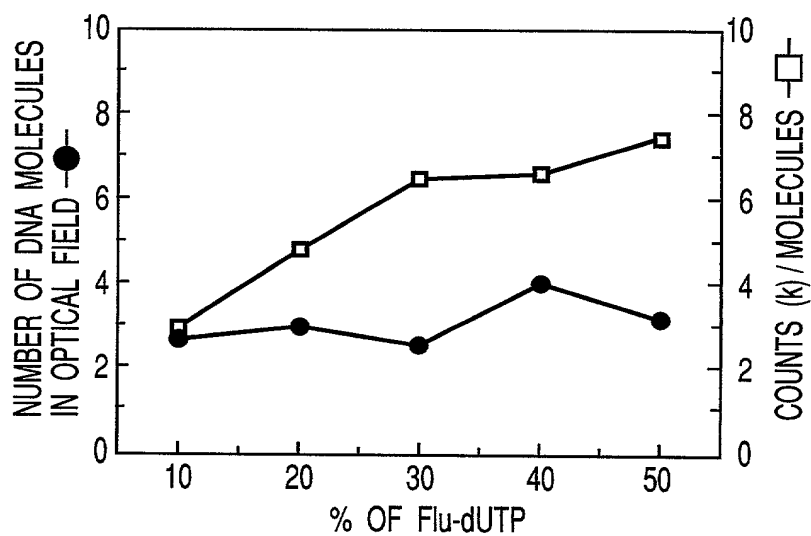


FIG. 5

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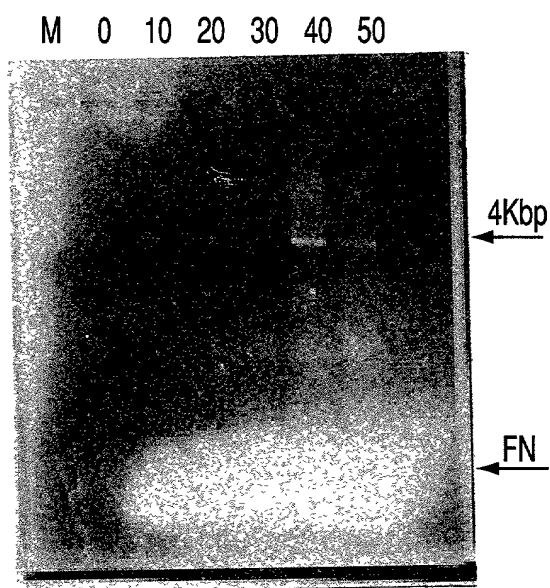


FIG. 4A

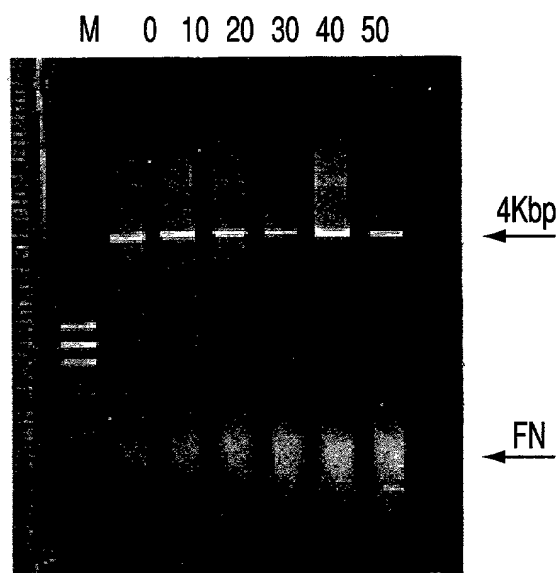


FIG. 4B

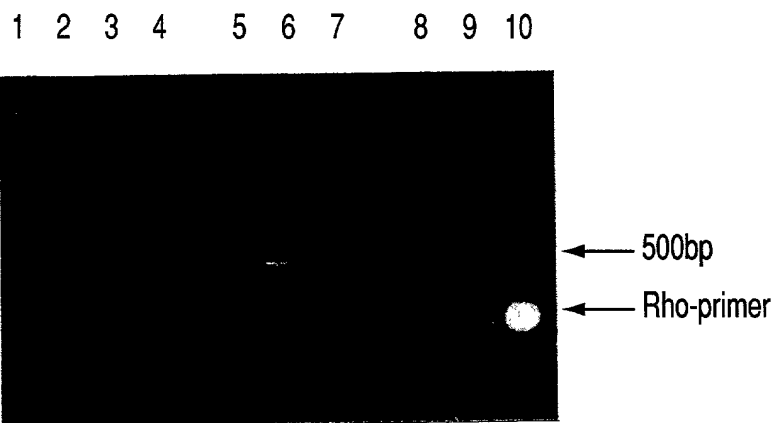


FIG. 11A

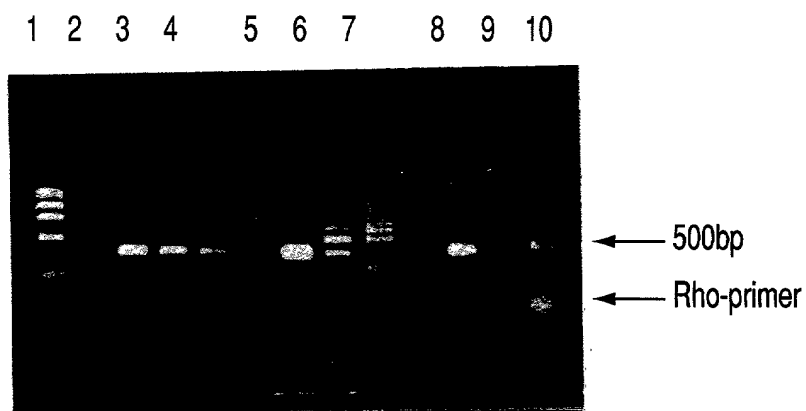


FIG. 11B

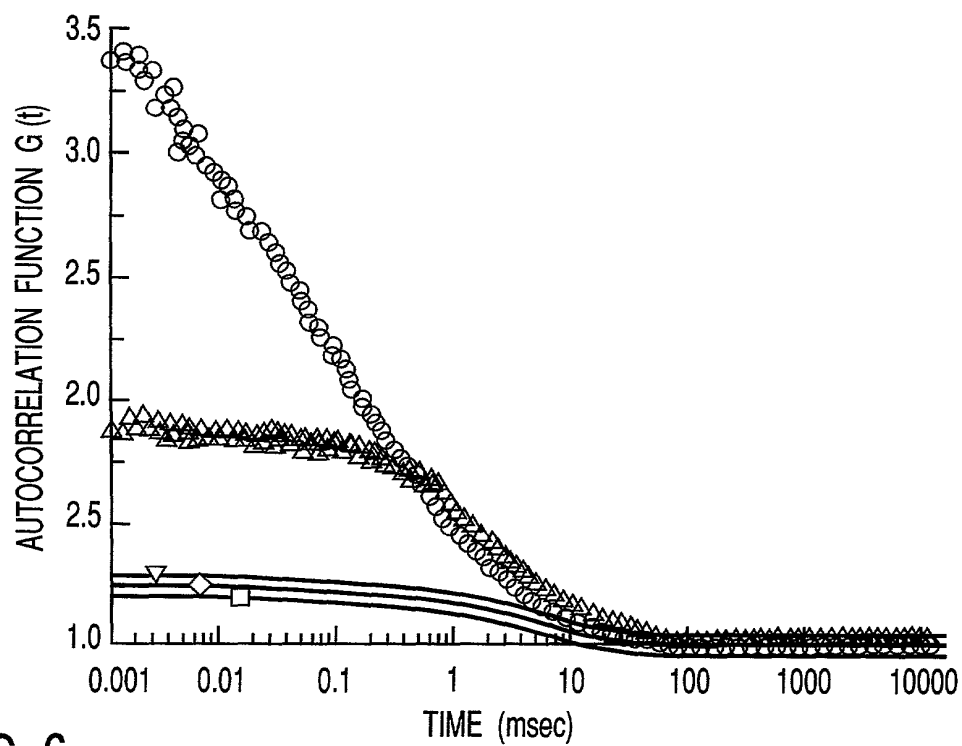


FIG. 6

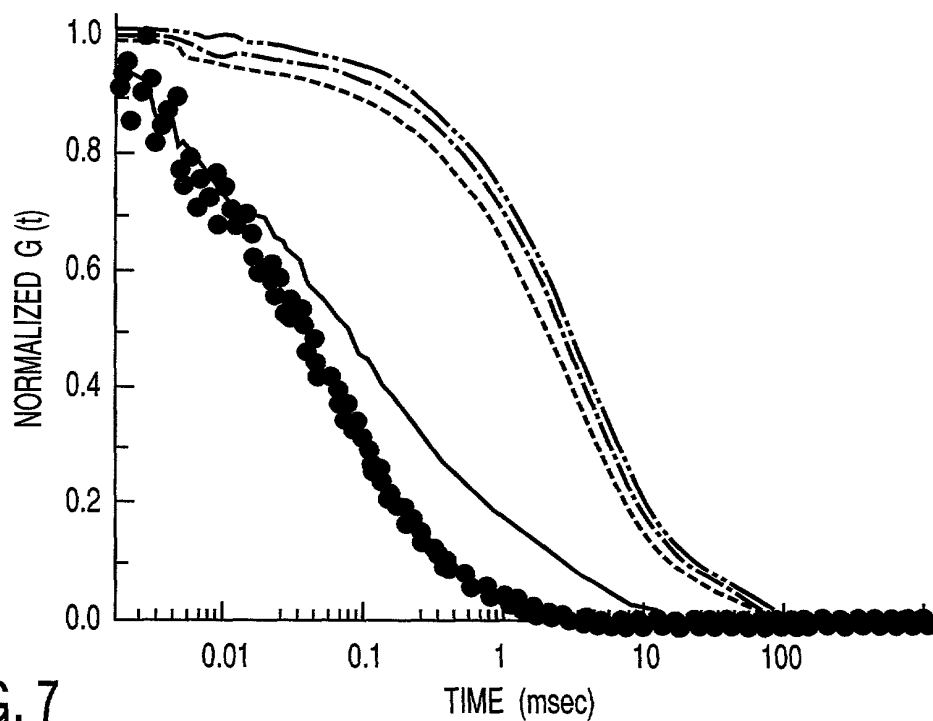


FIG. 7

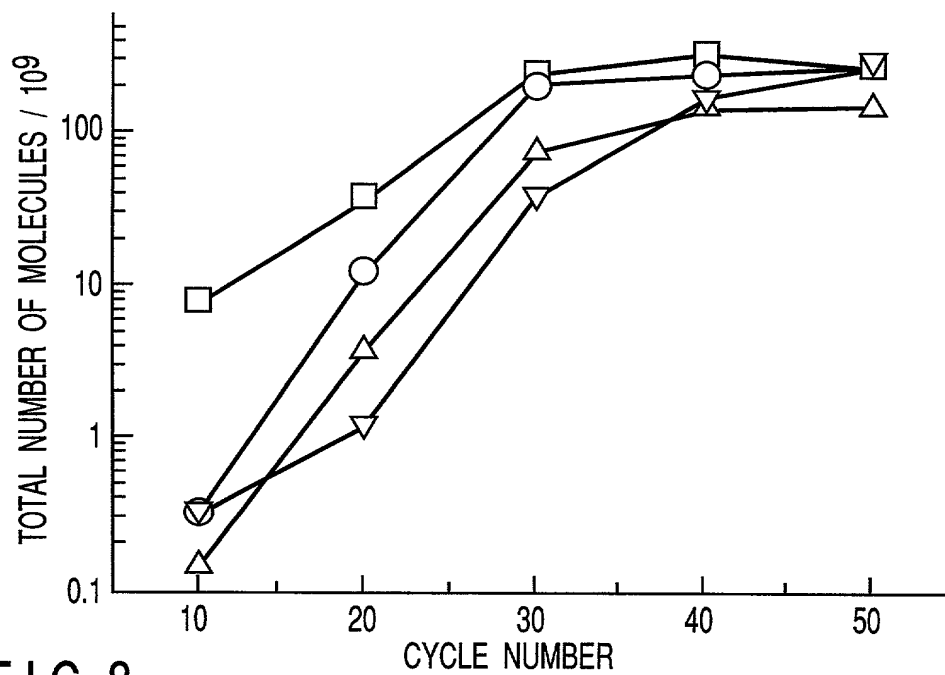


FIG. 8

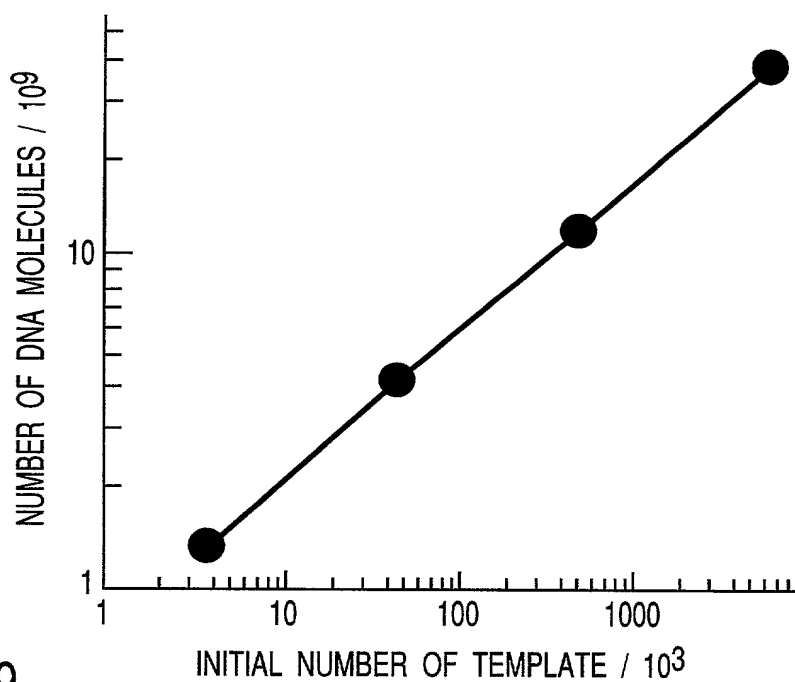


FIG. 9

FIG. 12

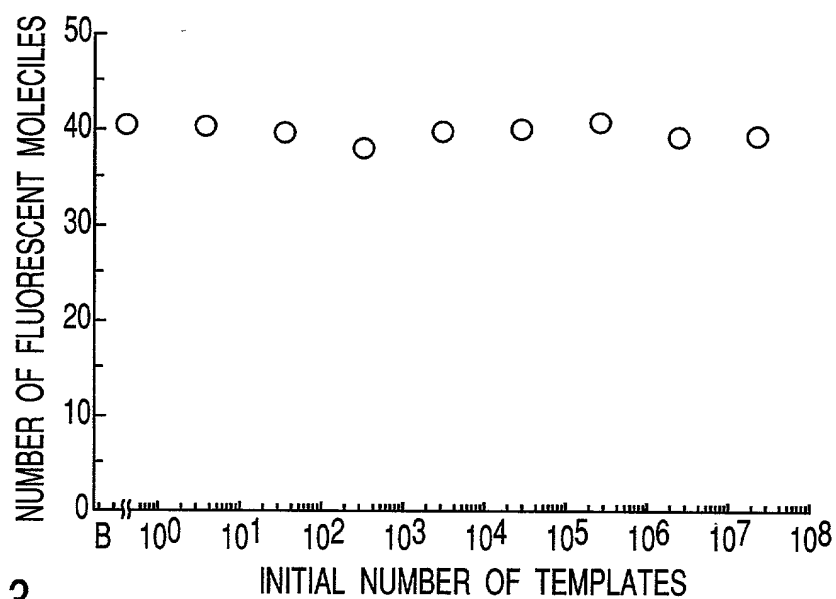


FIG. 13

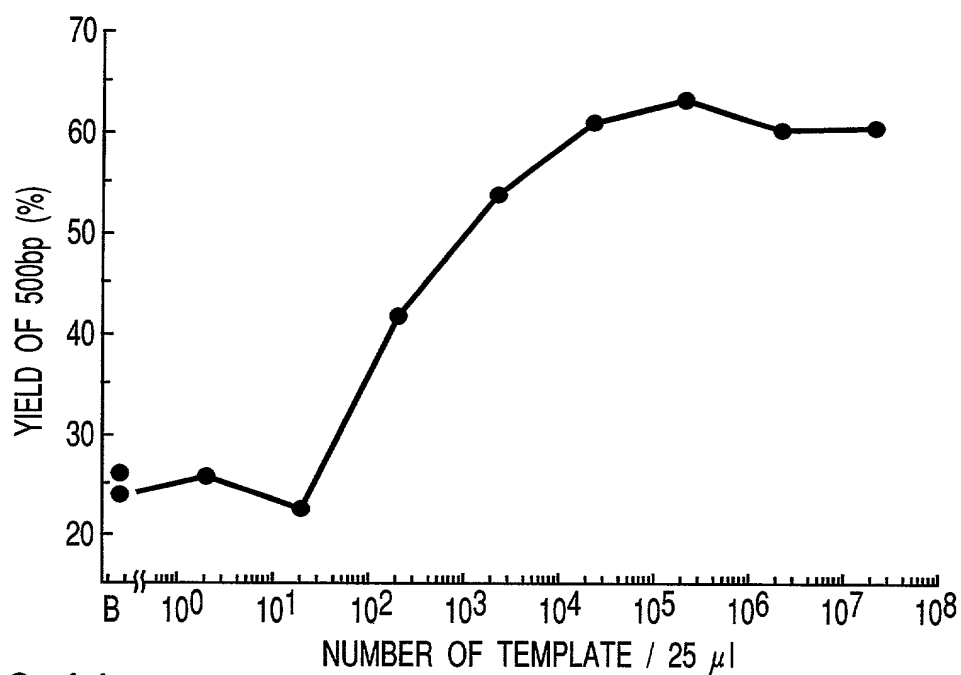


FIG. 14

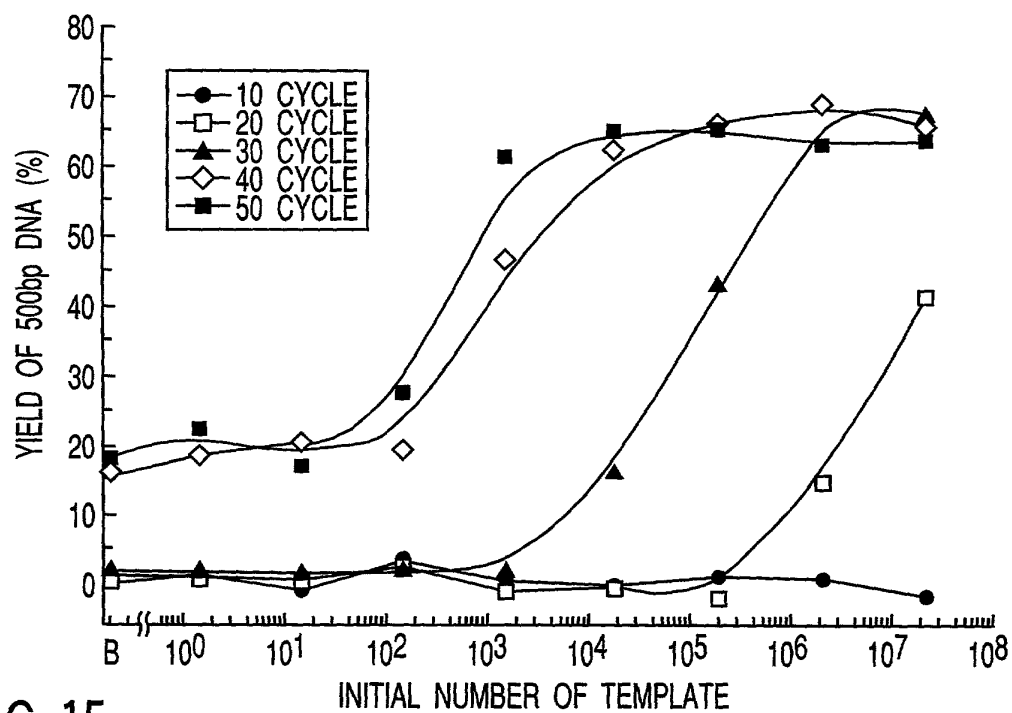


FIG. 15

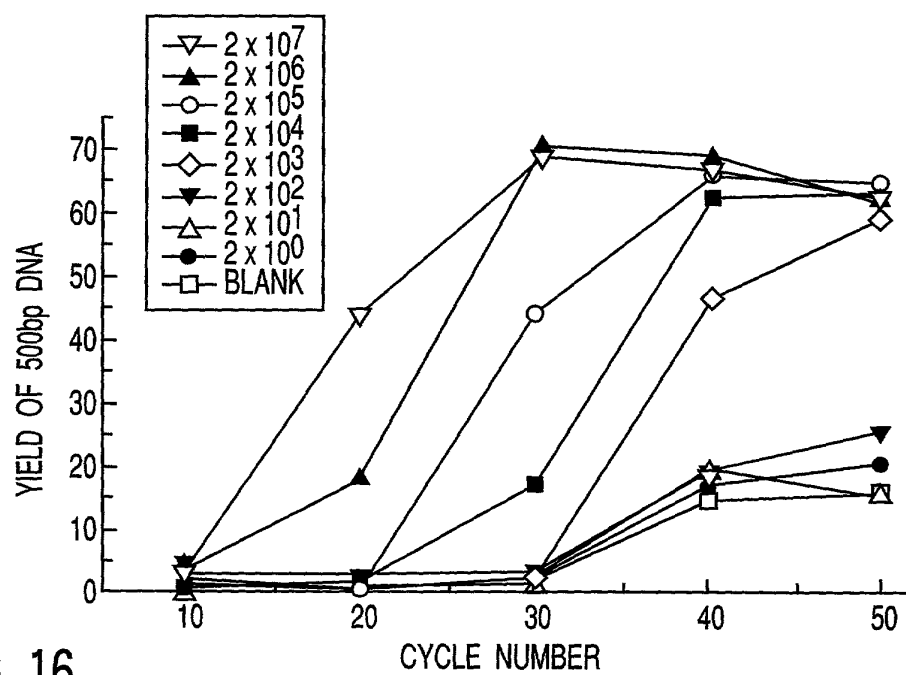


FIG. 16

Declaration Power of Attorney For Patent Application

特許出願宣言書及び委任状 Japanese Language Declaration 日本語宣言書

下記の氏名の発明者として、私は以下の通り宣言します。

As a below named inventor, I hereby declare that:

私の住所、私書箱、国籍は下記の私の氏名の横に記載された通りです。

My residence, post office address and citizenship are as stated below next to my name,

下記の名称の発明に関して請求範囲に記載され、特許出願している発明内容について、私が最初かつ唯一の発明者（下記の氏名が一つの場合）もしくは最初かつ共同発明者であると（下記の名称が複数の場合）信じています。

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

標的核酸の分析方法および装置

METHOD AND APPARATUS OF ANALYZING
TARGET NUCLEIC ACID

上記発明の明細書（下記の欄で×印がついていない場合は、本書に添付）は、

The specification of which is attached hereto unless the following box is checked:

☐ _____月 _____日に

提出され米国出願番号または特許協定条約

☐ was filed on _____
as United States Application Number or
PCT international Application Number

国際出願番号を _____ とし、

_____ and was amended on

（該当する場合） _____ 月 _____ 日に訂正されました。

_____ (if applicable).

私は、特許請求範囲を含む上記訂正後の明細書を検討し、内容を理解していることをここに表明します。

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

私は、連邦規則法典第37編第1条56項に定義されたとおり、特許資格の有無について重要な情報を開示する義務があることを認めます。

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56

Japanese Language Declaration

(日本語宣言書)

私は、合衆国法典第35編第119条(a)-(d)項又は第365条(b)に基づき下記の、米国以外の国の少なくとも一カ国を指定している特許協力条約365(a)項に基づく国際出願、又は外国での特許出願もしくは発明者証の出願についての外国優先権をここに主張するとともに、優先権を主張している、本出願の前に出願された特許または発明者証の外国出願を以下に、枠内をマークすることで、示しています。

I hereby claim foreign priority under Title 35, United States Code, Section 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)
外国での先行出願

Priority Not Claimed
優先権の主張なし

10-347493 (Number) (番号)	JAPAN (Country) (国名)	07/12/1998 (Day/Month/Year Filed) (出願年月日)	<input type="checkbox"/>
10-353254	JAPAN	11/12/1998	<input type="checkbox"/>
			<input type="checkbox"/>
			<input type="checkbox"/>
			<input type="checkbox"/>

私は、第35編米国法典119条(e)項に基づいて下記の米国特許出願規定に記載された権利をここに主張いたします。

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

(Application No.)
(出願番号)

(Filing Date)
(出願日)

(Application No.)
(出願番号)

(Filing Date)
(出願日)

私は、下記の米国法典第35編120条に基づいて下記の米国特許出願に記載された権利、又は米国を指定している特許協力条約365条(c)に基づき権利をここに主張します。また、本出願の各請求範囲の内容が米国法典第35編112条第1項又は特許協力条約で規定された方法で先行する米国特許出願に開示されていない限り、その先行米国出願書提出日以降で本出願書の日本国内または特許協力条約国際提出日までの期間中に入手された、連邦規則法典第37編1条56項で定義された特許資格の有無に関する重要な情報について開示義務があることを認識しています。

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) or 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT Information application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which become available between the filing date of the prior application and the national or PCT international filing date of application:

(Application No.)
(出願番号)

(Filing Date)
(出願日)

(Status: Patented, Pending, Abandoned)
(現況: 特許許可済、係属中、放棄済)

(Application No.)
(出願番号)

(Filing Date)
(出願日)

(Status: Patented, Pending, Abandoned)
(現況: 特許許可済、係属中、放棄済)

私は、私自身の知識に基づいて本宣言書中で私が行う表明が真実であり、かつ私の入手した情報と私の信じることに基づき表明が全て真実であると信じていること、さらに故意になされた虚偽の表明及びそれと同等の行為は米国法典第18編第1001条に基づき、罰金または拘禁、もしくはその両方により処罰されること、そしてそのような故意による虚偽の声明を行えば、出願した、又は既に許可された特許の有効性が失われることを認識し、よってここに上記のごとく宣誓を致します。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Japanese Language Declaration

(日本語宣言書)

委任状：私は、下記の発明者として、本出願に関する一切の手続きを米特許商標局に対して遂行する弁理士または代理人として、下記の者を指名いたします。
(弁理士、または代理人の氏名及び登録番号を明記のこと)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

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同発明者の署名 日付	Inventor's signature Date <i>Masataka Kinjo</i> May 20, 1999
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国 籍 日本	Citizenship JAPAN
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南新川公務員宿舎10-404	Sapporo-shi, Hokkaido, Japan

(第二以降の共同発明者に対しても同様に記載し、署名をすること。)

(Supply similar information and signature for second and subsequent joint inventors.)

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